

GLYCOSAMINOGLYCANS IN THE IRIDOCORNEAL ANGLE
OF THE NORMAL CANINE AND THE GLAUCOMATOUS BEAGLE

By

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Blessed, happy is the man who is patient under trial and stands up under temptation, for when he has stood the test and been approved he will receive the victor's crown.

James 1:12

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TABLE OF CONTENTS

| | <u>Page</u> |
|---|-------------|
| ACKNOWLEDGMENTS. | ii |
| LIST OF TABLES | v |
| LIST OF FIGURES | vi |
| ABSTRACT | ix |
| INTRODUCTION | 1 |
| REVIEW OF LITERATURE | 4 |
| POAG in the Beagle Model | 11 |
| Structure and Function of GAGs | 14 |
| GAG Profile of the Aqueous Outflow Pathway | 16 |
| Hypotheses | 19 |
| MATERIALS AND METHODS. | 21 |
| Experiment 1: Perfusion Study with Testicular Hyaluronidase. | 21 |
| Experiment 2: Isolation of GAGs From the Trabecular Meshwork, Sclera and Iris-Ciliary Body | 23 |
| Dissection of Anterior Segment | 24 |
| Isolation of Glycosaminoglycans. | 25 |
| Zone Electrophoresis of GAGs | 28 |
| Densitometry | 29 |
| Experiment 3: Enzymatic Degradation of Tissue GAGs . . | 29 |
| Experiment 4: Localization of GAGs in the Trabecular Meshwork by Histochemical Procedures | 31 |
| RESULTS. | 34 |
| Experiment 1: Perfusion Study with Testicular. | 34 |
| Hyaluronidase | |
| Experiment 2: Isolation of GAGs from the Trabecular Meshwork, Sclera and Iris-Ciliary Body | 35 |
| Experiment 3: Enzymatic Degradation of Tissue GAGs . . | 37 |
| Experiment 4: Localization of GAGs in the Trabecular Meshwork by Histochemical Procedures | 39 |
| DISCUSSION | 42 |
| SUMMARY. | 51 |
| APPENDIX | 52 |
| REFERENCES | 100 |
| BIOGRAPHICAL SKETCH. | 109 |

LIST OF TABLES

| <u>TABLE</u> | <u>Page</u> |
|---|-------------|
| 1. ANIMAL MODELS FOR GLAUCOMA COMPARED TO PRIMARY OPEN ANGLE GLAUCOMA IN HUMANS | 53 |
| 2. SPECIFIC ACTIVITY OF ENZYMES. | 55 |
| 3. PHYSIOLOGICAL DATA AND CLINICAL OBSERVATIONS ON THE GLAUCOMATOUS BEAGLE AND NORMOTENSIVE CANINE . . | 56 |
| 4. ANTERIOR SEGMENT DRY DEFATTED TISSUE WEIGHTS FROM THE GLAUCOMA AND AGE-MATCHED NORMAL EYES . . . | 57 |
| 5. MICROSPECTROPHOTOMETRY ANALYSIS OF EARLY AGE NORMAL AND EARLY GLAUCOMATOUS EYES. | 58 |
| 6. MICROSPECTROPHOTOMETRY ANALYSIS OF MODERATE AGE NORMAL AND MODERATE GLAUCOMATOUS EYES | 59 |
| 7. MICROSPECTROPHOTOMETRY ANALYSIS OF ADVANCED AGE NORMAL AND ADVANCED GLAUCOMATOUS EYES | 60 |

LIST OF FIGURES

| <u>FIGURE</u> | <u>Page</u> |
|--|-------------|
| 1. DISACCHARIDE REPEATING UNIT OF THE GLYCOSAMINOGLYCANS | 61 |
| 2. COMPOSITION OF THE GLYCOSAMINOGLYCANS AND SEQUENCE OF THE LINKAGE REGION | 62 |
| 3. PERFUSION SYSTEM DIAGRAM | 63 |
| 4. SCHEMATIC DIAGRAM OF THE ZEISS KONTRON SEM-IPS AND ZONAX SYSTEM | 64 |
| 5. NORMAL CANINE EYES PERFUSED 30 MINUTES WITH 0, 25, AND 50 I. U. OF HYALURONIDASE | 65 |
| 6. NORMAL CANINE EYES PERFUSED 30 MINUTES WITH 0, AND 100 I. U. OF HYALURONIDASE. | 66 |
| 7. GLAUCOMATOUS BEAGLE EYES PERFUSED 30 MINUTES WITH 0, 25 AND 50 I. U. OF HYALURONIDASE. | 67 |
| 8. NORMAL CANINE EYES PERFUSED 60 MINUTES WITH 0, 25, 50 AND 100 I. U. OF HYALURONIDASE. | 68 |
| 9. NORMAL CANINE EYES PERFUSED 60 MINUTES WITH 0 AND 100 I. U. OF HYALURONIDASE | 69 |
| 10. TRANSMISSION ELECTRON MICROGRAPH OF A NORMAL CANINE TRABECULAR BEAM, STAINED WITH COLLOIDAL IRON | 70 |
| 11. TRANSMISSION ELECTRON MICROGRAPH OF A NORMAL CANINE TRABECULAR BEAM, PERFUSED FOR 60 MINUTES WITH 100 I. U. OF HYALURONIDASE | 71 |
| 12. TRANSMISSION ELECTRON MICROGRAPH OF A TRABECULAR BEAM FROM AN ADVANCED GLAUCOMATOUS EYE, STAINED WITH COLLOIDAL IRON. | 72 |
| 13. TRANSMISSION ELECTRON MICROGRAPH OF A TRABECULAR BEAM FROM AN ADVANCED GLAUCOMATOUS EYE, PERFUSED FOR 30 MINUTES WITH 100 I. U. OF HYALURONIDASE . . . | 73 |

| | | |
|-----|---|----|
| 14. | NORMAL CANINE ANGLE. | 74 |
| 15. | MICROGRAPH OF A SAGITTAL SECTION OF THE SCLERA WITH THE TRABECULAR MESHWORK REMOVED. | 75 |
| 16. | CELLULOSE ACETATE ELECTROPHORESIS OF NORMAL TRABECULAR MESHWORK. | 76 |
| 17. | CELLULOSE ACETATE ELECTROPHORESIS OF EARLY GLAUCOMATOUS TRABECULAR MESHWORK | 77 |
| 18. | CELLULOSE ACETATE ELECTROPHORESIS OF MODERATE GLAUCOMATOUS TRABECULAR MESHWORK | 78 |
| 19. | CELLULOSE ACETATE ELECTROPHORESIS OF ADVANCED GLAUCOMATOUS TRABECULAR MESHWORK | 79 |
| 20. | CELLULOSE ACETATE ELECTROPHORESIS OF NORMAL IRIS-CILIARY BODY. | 80 |
| 21. | CELLULOSE ACETATE ELECTROPHORESIS OF EARLY AND MODERATE GLAUCOMATOUS IRIS-CILIARY BODY. | 81 |
| 22. | CELLULOSE ACETATE ELECTROPHORESIS OF ADVANCED GLAUCOMATOUS IRIS-CILIARY BODY | 82 |
| 23. | CELLULOSE ACETATE ELECTROPHORESIS OF ADVANCED GLAUCOMATOUS SCLERA. | 83 |
| 24. | CELLULOSE ACETATE ELECTROPHORESIS OF MODERATE GLAUCOMATOUS SCLERA. | 84 |
| 25. | DENSITOMETRY RECORDINGS OF CELLULOSE ACETATE MEMBRANES OF NORMAL, EARLY, MODERATE, AND ADVANCED GLAUCOMATOUS TRABECULAR MESHWORK. | 85 |
| 26. | DENSITOMETRY RECORDINGS OF CELLULOSE ACETATE MEMBRANES AFTER ISOLATED TM GAGs WERE EXPOSED TO HYALURONATE LYASE | 86 |
| 27. | DENSITOMETRY RECORDINGS OF CELLULOSE ACETATE MEMBRANES AFTER ISOLATED TRABECULAR MESHWORK GAGs WERE EXPOSED TO CHONDROITIN ABC LYASE, HEPARITINASE, HEPARINASE AND KERATANASE. | 87 |
| 28. | DENSITOMETRY RECORDINGS OF CELLULOSE ACETATE MEMBRANES OF NORMAL AND GLAUCOMATOUS IRIS-CILIARY BODY BEFORE AND AFTER HYALURONATE LYASE. | 88 |

| | | |
|-----|---|----|
| 29. | DENSITOMETRY RECORDING OF CELLULOSE ACETATE MEMBRANES OF NORMAL AND GLAUCOMATOUS IRIS-CILIARY BODY AFTER CHONDROITIN ABC LYASE, HEPARITINASE, HEPARINASE AND KERATANASE. | 89 |
| 30. | DENSITOMETRY RECORDINGS OF CELLULOSE ACETATE MEMBRANES OF NORMAL AND GLAUCOMATOUS SCLERA BEFORE AND AFTER HYALURONATE LYASE. | 90 |
| 31. | DENSITOMETRY RECORDING OF CELLULOSE ACETATE MEMBRANES OF NORMAL AND GLAUCOMATOUS SCLERA AFTER CHONDROITIN ABC LYASE, HEPARITINASE, HEPARINASE AND KERATANASE | 91 |
| 32. | CANINE IRIDOCORNEAL ANGLE STAINED WITH ALCIAN BLUE . | 92 |
| 33. | TRABECULAR MESHWORK BEAMS STAINED WITH ALCIAN BLUE . | 93 |
| 34. | MICROGRAPH OF THE EARLY AGE NORMAL IRIDOCORNEAL ANGLE GENERATED BY THE ZEISS IMAGE PROCESSING SYSTEM | 94 |
| 35. | MICROGRAPH OF THE EARLY GLAUCOMATOUS IRIDOCORNEAL ANGLE GENERATED BY THE ZEISS IMAGE PROCESSING SYSTEM | 95 |
| 36. | MICROGRAPH OF THE MODERATE AGE NORMAL IRIDO- CORNEAL ANGLE GENERATED BY THE ZEISS IMAGING PROCESSING SYSTEM. | 96 |
| 37. | MICROGRAPH OF THE MODERATE GLAUCOMA IRIDOCORNEAL ANGLE GENERATED BY THE ZEISS IMAGE PROCESSING SYSTEM | 97 |
| 38. | MICROGRAPH OF THE ADVANCED AGE NORMAL IRIDO- CORNEAL ANGLE GENERATED BY THE ZEISS PROCESSING SYSTEM | 98 |
| 39. | MICROGRAPH OF THE ADVANCED GLAUCOMA IRIDOCORNEAL ANGLE GENERATED BY THE ZEISS IMAGE PROCESSING SYSTEM | 99 |

Abstract of Dissertation Presented to the Graduate School of
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The literature substantiates the importance of glycosaminoglycans (GAGs) in 1) regulating normal intraocular pressure (IOP) and 2) a contributing factor of increased IOP in primary open angle glaucoma (POAG) of humans and canines. Glycosaminoglycans may biochemically change during the course of the disease which impairs the aqueous outflow through the trabecular meshwork (TM) of the eye. The glaucomatous Beagle was used in this study because it is a suitable model for studying the biochemical and physiological mechanisms of POAG.

To determine the importance of the GAGs in the iridocorneal angle, testicular hyaluronidase (a GAG degrading enzyme) was perfused into the anterior chamber of normal canines and glaucomatous beagles. The results indicated that in all perfused concentrations the enzyme significantly increased aqueous outflow from the eye in the normal dogs but the enzyme did not change aqueous outflow in glaucomatous Beagles. After perfusion, the anterior segment and the aqueous out-

flow pathway were prepared for electron microscopy and stained with colloidal iron. Glaucomatous eyes contained a hyaluronidase resistant material, primarily located in the intertrabecular spaces.

In separate studies, the TM, iris-ciliary body and sclera in early, moderate and advanced stages of POAG in the Beagle and in age-matched control dogs were analyzed for GAGs. The GAG fraction was isolated by chloroform:methanol dilipidation, pronase digestion and selective ethanol precipitation. The enriched - GAG fraction was subjected to deoxyribonuclease I and ribonuclease A in order to remove nucleic acids. A purified GAG fraction was obtained by size exclusion high performance liquid chromatography. The GAGs were identified and characterized by zone electrophoresis using cellulose acetate membranes and by specific GAG-degrading enzymes. By use of histochemical techniques, with a computer-aided microspectrophotometer and video image processing system, GAGs were identified in the TM and juxtacanalicular zone of normal and glaucomatous eyes. GAGs of the normal and early glaucomatous TM were hyaluronic acid, heparan sulfate and chondroitin-dermatan sulfate, a GAG profile similar to that of the human TM. In moderate and advanced stages of POAG in Beagles, the profile was hyaluronic acid and an unidentified GAG moiety. The unidentified component, which represents an enzyme-resistant GAG or a glycopeptide, was the major component of the TM in advanced POAG.

From these studies, an enzyme-resistant GAG was isolated from the TM of glaucomatous eyes, which is not present in the normal. The biochemical analysis of this material may elucidate the pathogenesis of POAG in humans and canines.

INTRODUCTION

Glaucoma is characterized by an increase in intraocular pressure (IOP), which causes visual impairment and blindness. The disorder is divided clinically into three major groups: primary (which is subclassified as open, narrow and closed iridocorneal angle), secondary and congenital. Primary open angle glaucoma (POAG) is the most common type and does not have any recognized factors which would account for the increased IOP, whereas the secondary and congenital types are caused by concurrent ocular conditions such as iridocyclitis, neoplastic disease and, in congenital glaucoma, goniodysgenesis. POAG in the human and the canine constitutes approximately 70% of all the cases of glaucoma and is a leading cause of blindness (33, 51). It is, therefore, imperative that an understanding of the basic disease mechanism be gained and a more suitable means of treating glaucoma be found than is presently available.

Intraocular pressure is a balance of aqueous humor production and its outflow from the eye. Aqueous humor is produced by the ciliary body and flows through the pupil into the anterior chamber. It leaves the eye by way of the corneoscleral trabecular meshwork (TM) and enters the collecting veins (comprising the angular aqueous plexus, which empties into the larger intrascleral venous plexus) and posteriorly through the uveoscleral pathway. The latter is classified as

unconventional outflow (10, 11, 23, 36, 90).

Uveoscleral outflow is similar in humans and in the canine, amounting to 4-14% in humans and to 15% of the total outflow in the normal dog. In contrast, the glaucomatous Beagle has a marked reduction in uveoscleral outflow, amounting to only 3% of the total outflow (10, 14, 15, 17, 71). Nonhuman primates have uveoscleral outflow amounting to 30-65% of the total, indicating that these animals are less desirable as models for studying POAG (14, 15, 17, 71).

The corneoscleral TM, the conventional outflow pathway, consists of flat perforated sheets. These are similar to the outer layers of the uvea; the sheets are parallel to the limbal structures. Over several years a number of investigators have concluded that trabecular sheets in the primate consist of a collagen core, elastic-like tissue, a cortical zone and endothelial cells (85, 95, 107). The orientation of the core collagen fibers (parallel to the limbus) appears to be related to the function of collagen fibrils and the pull associated with the contraction of the longitudinal and radial ciliary muscles. The elastic-like tissue apparently provides the trabeculae with some degree of resiliency (25). The cortical zone is located between the endothelium and the collagen-elastic components. The predominate material in this region is the basement membrane of the endothelial cells.

In humans, during the normal aging process, collagen fibers undergo a conformational change; they appear thicker and less compact, containing "curly" collagen (short sections, densely arranged colla-

gen). The cortical zone also appears to increase in thickness during aging which is mainly due to the increased amount of basement membrane material (25). Attached to the basement membrane of the cortical zone are the endothelial cells, which produce the basement membrane. Additional anchorage for the endothelial cells is provided by hemidesmosomes, which project down onto the basal region. All trabecular spaces and interconnecting tortuous pathways are lined by endothelial cells in primates (95).

The role of the ciliary muscle in reducing the resistance to aqueous outflow has been known for a long period of time (95). During accommodation and under the influence of parasympathomimetic drugs, the TM tends to expand, thus facilitating aqueous outflow (28, 85, 95). However, aqueous outflow cannot be accounted completely by the porosity of the trabecular sheets, therefore, some other mechanism must exist (7).

REVIEW OF THE LITERATURE

Over the past few years a number of theories have been described for the normal conventional outflow pathway and the pathological changes which occur in the POAG eyes of man.

Aqueous outflow is pressure-dependent according to Poiseuille's law, which defines the relationship between IOP, intraocular volume and aqueous flow rate.

$$\text{Poiseuille's equation: } F = \frac{(P_1 - P_2) \pi r^4 n}{8 \eta l}$$

The flow (F) is proportional to the pressure difference ($P_1 - P_2$) from one end to the other (also called the pressure head between the ciliary body processes and the TM, and is also proportional to n and to r^4 (the number and the radius of pores in the TM to the fourth power connecting the two ends of the system). The length of the tubes (l) and viscosity (η) of the fluid in a system must also be considered; flow would be inversely proportional to these two factors. The rate of aqueous outflow can then be linearly related to the pressure gradient; this has been confirmed experimentally (8, 24, 41, 77).

Tripathi noted in human eyes that endothelium of the collecting channels undergo a vacuolation cycle which allows for aqueous humor to cross from the TM into the canal of Schlemm (96). The vacuolation

cycle was theorized to be a factor to aqueous outflow in the corneo-scleral TM. It is known that the pressure gradient between the IOP and Schlemm's canal is only a few millimeters of mercury (mmHg) which probably does not cause vacuolation unless the endothelial cells possess some unusual cellular characteristic (25).

The endothelial cells lining Schlemm's canal are limited in actively transporting large vacuoles of aqueous, this implies that some other mechanism must be involved in regulating aqueous outflow. Cholinergic and adrenergic innervation of the ciliary body and aqueous outflow pathway has been established but nerve endings have not been isolated in the endothelial lining of Schlemm's canal (25, 46). Fluorescent histochemical techniques showed that adrenergic nerve fibers were present in the subepithelial portions of the ciliary body with extensions innervating the stroma of the ciliary body processes. Fibers were also present in the aqueous sinus plexus and less extensively in the ciliary body musculature (25, 46). Cholinergic fibers (identified by the thiocholine method for cholinesterase and the Mindel and Mittag method for choline acetyltransferase, 46) were located in the ciliary body, ciliary processes, ciliary musculature and epithelial cells. Low levels of cholinergic activity were found in the TM and associated outflow channels. In the advanced glaucomatous Beagle the adrenergic and cholinergic innervation of the ciliary body and aqueous outflow pathway appears to be less extensive than the normal (25, 46). This decrease of neuronal activity may be second-

dary to the disease process. Aqueous humor outflow in other species (such as nonhuman primates, rabbits, dogs, and birds) has been shown to be dependent on a pressure gradient and to have a vacuolation cycle (16, 47, 49, 77, 84, 89, 97-99).

Morphological and ultrastructural studies of human iridocorneal angles have indicated that the ciliary muscle tendons are connected to a special subendothelial network of elastic-like fibers, which in turn are connected by fine fibers to the endothelial cells of Schlemm's canal, the juxtacanalicular region and outer corneoscleral TM. Thus the patterns of the aqueous humor outflow are most likely influenced by the action of the ciliary musculature (84). This is substantiated by the mechanism of action of many parasympathomimetic miotics, such as pilocarpine, which produces direct stimulation of the iris and ciliary musculature (47, 49).

Rohen described an elastic-like fibrous material in the juxtacanalicular zone (cribriform plexus) which increased in aging eyes, as well as in POAG (84, 86). The elastic-like material was classified in three types: Type I a low electron-dense material, Type II a high electron-dense material found in patches throughout the cribriform layer, and Type III, less dense than Type II but containing clusters of banded material with a specific periodicity. In the glaucomatous eyes, the predominate type was Type III plaques, which were observed in the juxtacanalicular zone. Type II material is usually embedded within the Type III plaques; both were more prevalent in the glauco-

matous than in the normal eye (84). Although the cause of the extracellular formation of electron-dense material is unknown, Rohen hypothesized that it may be a change in the pattern of GAGs secreted by the juxtacanalicular zone (69, 84, 86).

Alvarado investigated the cellular changes with age in the human TM. A loss of cellularity (cells/unit tissue area) and cell numbers were found with increasing age (3), which were part of the normal aging process. A loss of 0.58% cells per year apparently occurs after the first ten years of life (2). In POAG, the loss of cellularity is greater (2, 72). The data suggested that POAG patients have fewer cell numbers at birth, are less tolerant to increased ocular hypertension and the inner TM is more susceptible to cell loss (2, 42). The loss of cellularity is prevalent in the canine corneal endothelium as well, indicating that both trabecular and corneal cells may have limited regenerative capability (48).

Another factor to consider is cellular loss due to the effects of glaucoma medication and the advanced disease state. An early study indicated that the cell loss is secondary to the effect of glaucoma (42). However, Alvarado's data showed no significant differences in cellularity between those on medication and those who did not receive any antiglaucoma therapy (2).

Johnson and Kamm examined the resistance to aqueous humor flow in the juxtacanalicular meshwork and noted that pore size alone was not sufficient to account for the total resistance. Using a mathematical

model to predict the resistance of the juxtacanalicular region, the calculated value for the normal human eye was $0.016 \mu\text{l}^{-1}\text{min}^{-1}\text{mmHg}$. This is well below the $2-10 \mu\text{l}^{-1}\text{min}^{-1}\text{mmHg}$ measured outflow resistance of the normal eye. Johnson concluded that what appeared to be open spaces in the meshwork observed in the transmission electron microscopy (TEM) micrographs might contain materials such as GAGs and other glycoproteins, which are not visible by routine electron microscopy (57).

Johnson measured the flow of aqueous humor through micro-porous filters having pore size and flow dimensions similar to those of the juxtacanalicular zone. Bovine and nonhuman primate aqueous humor and isotonic saline were passed through polycarbonate filters to determine flow resistance. The results indicated that aqueous humor had a greater resistance to flow than normal saline; this could be attributed to proteins or glycoproteins in aqueous humor (56). The aqueous humor (from Bovine and nonhuman primates) were subsequently subjected to papain and testicular hyaluronidase since aqueous humor is composed of small amounts of protein ($100-600 \mu\text{g/ml}$) and GAGs ($1-4 \mu\text{g/ml}$) (50, 67). Papain degradation of aqueous humor resulted in an increase of aqueous flow through the polycarbonate filters and hyaluronidase treatment did not significantly change the aqueous outflow. The authors concluded that proteins or glycoproteins play an important role in blocking the porous channels of the juxtacanalicular meshwork (28, 56) and that the "wash-out" effect (which occur when the eye is

perfused over a period of time) is the result of the elimination of these glycoproteins from the aqueous outflow pathway (55). One of the major problems with this study is that the filtration system does not represent an in vivo system and does not represent the cell biology of the juxtacanalicular zone. The micro-porous filters used in these studies may interact with aqueous humor, causing the proteins to bind to the filter system.

Fibronectin, an extracellular glycoprotein, is produced by the endothelial cells of the TM (29, 79). Fibronectin is located in the subendothelium of Schlemm's canal. This suggests that fibronectin is in close association with the GAGs, which are localized in greater amounts on the inner wall of Schlemm's canal (30). In a recent study, fibronectin seemed to be associated with the basement membrane (29, 106) and the cell surfaces of the juxtacanalicular channels (29).

Other immunohistological studies have indicated that fibronectin, collagen Types I-IV, VI and laminin are located in the subendothelial regions of the TM and Schlemm's canal. These compounds have also been reported to be more numerous in glaucomatous eyes than in age-matched normals (29, 83). However, another study noted only subtle differences between glaucomatous eyes and normal eyes of humans in the distribution of collagen Types IV and VI (1). Cell culture studies have indicated that fibronectin has a higher binding affinity for collagen Types I and III, which may play a role in the disease process of glaucoma (105). Plasma fibronectin has been shown in patients with

glaucoma to have a higher binding affinity for interstitial collagens than for the basement membrane, collagen Type IV (104). This may not be the case in the juxtacanalicular region itself.

It has been proposed that GAGs play a major role in the regulation of IOP. Earlier studies have demonstrated that GAGs are present in the iridocorneal angle of a number of species. Barany infused Bovine testicular hyaluronidase (an enzyme specific for degrading hyaluronic acid and some of the chondroitin sulfates) into the anterior chamber of a number of animal species and noted a substantial increase in aqueous humor outflow (6). Similar results were noted in the TM of human eyes using histochemical techniques (109). Armaly and Wang, using colloidal iron stain for GAGs in the TM of normal monkeys (Rhesus), demonstrated the presence of GAGs in the basement membrane of endothelium, intertrabecular spaces, and in the ground substance and basement membrane of the endothelium of the canal of Schlemm (4).

Richardson found that GAGs are localized in the TM of cats' iridocorneal angle. In this study ruthenium red stain was used to localize and characterize glycoconjugates. Testicular hyaluronidase, neuraminidase and papain were used to determine the composition of the ruthenium red stain material. The GAGs sensitive to testicular hyaluronidase were located on the endothelial cell surface in the TM and in the amorphous tissue of the trabecular beams (80). Collagen and elastic tissue in the TM also stained with ruthenium red because

of the carbohydrate moieties. Sialoglycoproteins, sensitive to neuraminidase degradation, were localized on the luminal surface of the endothelial cells of the aqueous plexus (Schlemm's canal in humans). Papain digestion demonstrated that GAGs in the connective tissue were sensitive to this enzyme, but the endothelial cell GAGs were not. Richardson concluded that connective tissue GAGs were complexed to proteins whereas endothelial GAGs were not (80). However, it has been shown that cell surface endothelial GAGs have protein moieties (53).

POAG in the Beagle Model

In the Beagle, POAG is inherited as an autosomal recessive trait; it can also be inherited as an autosomal recessive trait in humans (12, 31, 38, 101). Occasionally in humans, POAG is inherited as an autosomal dominant trait with a variable penetrance (31).

Normal IOP in the canine, as measured by applanation tonometry, is approximately 21 mmHg; IOP in the preglaucomatous Beagle is the same. As the disease progresses, IOP increases to an average of 28 mmHg for an animal in the moderate stage of glaucoma and ranges between 30 to 50 mmHg in the advanced stage of the disease (35). Similar changes are seen in human POAG (51).

Tonography, which measures the outflow of aqueous humor from the eye, is expressed as a coefficient of aqueous humor flow (C-value) and has a mean coefficient of 0.24 (S.D. ± 0.07) $\mu\text{l}^{-1}\text{min}^{-1}\text{mmHg}$ in the normal

canine. In the early glaucomatous dog the coefficient of aqueous outflow decreases to an average of 0.13 (S.D.+ 0.05) and 0.07 (S.D.+ 0.03) in the moderate glaucomatous dog (37).

Gonioscopically, Beagles with POAG have an open iridocorneal angle in the early stage of the disease, open to narrow in the moderate stage and narrow to closed during the advanced stage. The gonioscopic condition of the advanced disease state is similar to chronic narrow angle glaucoma in humans.

Other changes associated with POAG in the Beagle include optic disc cupping with eventual atrophy, buphthalmia, cataract formation, vitreous syneresis and, in the latter stages of the disease, phthisis bulbi (34, 100).

The literature substantiates that diurnal variation in IOP and adrenal corticosteroid function are related. Humans patients with POAG exhibit abnormalities in corticosteroid metabolism. A relationship between plasma 17-hydroxycorticosteroids (cortisol) values and diurnal IOP measurements (minimal and maximal values) has been established (101). A similar relationship has been documented in the glaucomatous Beagle; where serum cortisol values were significantly higher in the Beagle with POAG than in the normal dog (20).

A number of animal models have been used over the past several decades to study POAG. The New Zealand rabbit, which has a spontaneous glaucoma, has been studied for two decades. The pathogenesis of this disease has been associated with congenital goniodysgenesis (66).

Other models for glaucoma, such as experimentally induced glaucoma in the rabbit and nonhuman primate have been utilized with limited success and with varied results. The avian eye has also been considered as a model for glaucoma. The buphthalmia of chickens, which is photoinduced, is characterized by a narrow iridocorneal angle, suggesting angle closure (103). The domestic turkey (Mellea-gris gallapavo) has been shown to have an hereditary eye defect. This defect is caused by a completely penetrant, incompletely dominant, autosomal gene with variable expressivity. The disorder is characterized by a progressive posterior synechia which leads to a secondary angle closure (26). The turkey model is representative of secondary glaucoma and appears not to be suitable as a model for POAG. Table 1 compares the physiological and pharmacological parameters of various animal models to those of human POAG. The POAG in humans and Beagles are similar in the type of glaucoma, pathogenesis and clinical course of the disease. Other similarities included: changes in IOP (which increases over the course of the disease), episcleral venous pressure, axoplasmic flow, elevated blood cortisol levels, a decrease in tonography C-values and a positive correlation with the pharmacological agents used to treat POAG in humans. It is evident from the data in Table 1 that the Beagle is the animal model that most closely resembles human POAG.

Structure and Function of GAGs

Glycosaminoglycans appear to be a contributing factor in maintaining normal IOP and in POAG.

Macromolecules that contain both carbohydrates and proteins are classified as glycoproteins or as proteoglycans. A glycoprotein molecule is composed of a protein chain, consisting of approximately 200 amino acid units, to which carbohydrate moieties are covalently attached. Carbohydrate moieties consist of oligosaccharide chains that are usually branched. Proteoglycans also contain protein cores, but the carbohydrate moieties form a linear chain with characteristic disaccharide repeating units. Another feature that distinguishes glycoproteins from proteoglycans is the number of carbohydrate units per protein core. In glycoproteins, the protein moieties vary considerably (15% to 95% of the molecular weight) whereas the carbohydrate moieties predominate in proteoglycans; in some cases they comprise 95% of the molecular weight (58, 102). Essentially GAGs are the carbohydrate subunits of proteoglycan. The GAGs are long chain, polyanionic molecules. Sulfate and carboxyl groups are usually associated with carbohydrates. In tissue, GAGs usually occur as proteoglycans with several polysaccharide chains attached to the protein core (53).

There are six major classes of GAGs (hyaluronic acid, chondroitin sulfates, dermatan sulfate, keratan sulfate, heparan sulfate and

heparin); they are distinguished by their carbohydrate composition and primary structure (Figure 1). Hyaluronic acid, which contains disaccharide repeating units of glucuronic acid and N-acetyl-glucosamine, is the largest with a molecular weight of approximately 1×10^7 . Chondroitin sulfates are classified as A and C or 4- and 6- sulfates, respectively, depending on the location of the sulfate ester. The chondroitin sulfates usually have a molecular weight between 1 and 6×10^4 . Dermatan sulfate is similar to chondroitin 4-sulfate with the exception of an iduronic acid in place of the glucuronic acid moiety. Keratan sulfate disaccharide units are composed of galactose and N-acetyl-glucosamine, as well as fucose, sialic acid and mannose. The keratan sulfate isolated from the cornea is subclassified as Type I, with a N-acetyl-glucosaminyl-asparaginyl linkage to the protein core. Type II keratan sulfate is attached to a protein through N-acetyl-galactosamine by an O-glycosidic linkage with either threonine or serine; it has been isolated from cartilage and bone. Heparan sulfate and heparin have repeating units of glucuronic acid 2-sulfate or iduronic acid and N-acetyl-glucosamine 6-sulfate. The difference between the two is that heparan sulfate has fewer sulfate groups and fewer iduronic acid units. Heparin and heparan sulfate also are the smallest GAGs with a molecular weight of 1×10^4 (53, 102). The linkage and sequence of the heteropolysaccharide groups are illustrated in Figure 2.

Glycosaminoglycans have a number of important biological functions but many functions still remain unknown. Because of their polyanionic nature, these macromolecules appear to influence aqueous humor dynamics (movement of water and solutes through the extracellular matrix). Grierson noted that the GAGs in the basal lamina may influence the rate of formation of giant vacuoles within the endothelial cells of Schlemm's canal (22, 44). The GAGs have a number of other functions within connective tissue, including regulation of cell metabolism, lubrication, maintenance of structural integrity, remodeling and wound healing (22, 73, 94). Glycosaminoglycans have also been postulated to play a role in cell to cell and cell-substrate interactions. According to some studies, cell associated GAGs may act as receptors for circulating biochemical components (53).

GAG Profile of the Aqueous Outflow Pathway

Knepper, using zone electrophoresis elucidated the distribution of GAGs within the iridocorneal angle, iris-ciliary body, and sclera of the New Zealand Red rabbit. These biochemical studies indicated that the primary GAGs of the sclera are hyaluronic acid, chondroitin sulfate and dermatan sulfate-chondroitin sulfate whereas the TM and iris-ciliary body GAGs are hyaluronic acid, keratan sulfate, heparan sulfate and conjugates of dermatan sulfate-chondroitin sulfate 4- and 6- (64). Studies involving rabbit and human TM tissue indicate that

hyaluronic acid and dermatan-chondroitin sulfate are the major GAG components; keratan sulfate and heparan sulfate are present in smaller quantities (63, 64). The GAGs of the aqueous outflow pathway most likely modulate the aqueous humor flow since proteoglycans have been demonstrated to form highly viscous gel-like compounds which immobilize the flow of water (13).

Perfusion of rabbit eyes with Streptomyces hyaluronidase and Bovine testicular hyaluronidase (using zone electrophoresis and densitometry techniques to analyze the GAGs) revealed that, at physiological pH's, Streptomyces hyaluronidase was ten times more effective in increasing aqueous outflow than was testicular hyaluronidase (65). This study also indicated that hyaluronic acid is important in resistance to aqueous humor outflow in normal eyes.

The cells of the TM secrete not only GAGs but also glycopeptides (64). The biosynthesis of both occurs in the endoplasmic reticulum and Golgi apparatus (glycosylation) of secretory cells such as the chondrocytes of cartilage. Monosaccharide units are added to carbohydrate chains by transferring them from various uridine diphosphate sugars and by the sequential action of a series of glycosyl transferase enzymes (81, 102). Knepper evaluated the synthesis of GAGs and glycopeptides by using radio labeled GAG precursors ($[^3\text{H}]$ glucosamine and $[^{35}\text{S}]$ sulfate) and measuring their incorporation into anterior segment tissue (62). The results of this study indicated that all anterior segment tissues were active in incorporating GAG

precursors, with the iris-ciliary body having the highest rate of synthesis followed by the TM and the anterior sclera. The gel filtration chromatography profile demonstrated the presence of synthesized long-chain GAG, as well as recently formed precursors of GAGs and glycopeptides (62). These results were similar to those of cell culture studies in which TM cells lines were used. The incorporation of [^{35}S] sulfate and [^{14}C] glucosamine precursors into trabecular cell culture explants (from Saimiri monkey) indicated that 63% of the GAGs were hyaluronic acid, 6% chondroitin sulfate and 31% dermatan sulfate (32, 91).

The effects of aging and glucocorticoids on the GAGs of ocular tissue have been studied. Knepper analyzed the effects of dexamethasone on the eyes of young and aged rabbits. The GAG moieties were analyzed from the central and peripheral corneal, scleral and iris tissue. Dexamethasone caused an increase in IOP in the young (8 weeks of age) animals but had no effect in older rabbits, 3 years of age (60). Changes in GAG distribution were noted as functions of age and dexamethasone treatment. Comparing younger rabbit eyes to the older age group, the GAG ratio of keratan sulfate to uronic acid decreased with increasing age. In the steroid-treated groups, the older rabbits showed an increase in the keratan sulfate to uronic acid GAG ratio over the younger animals (60). This suggests the change in the distribution of GAG moieties and the water-binding characteristics of GAGs are responsible for the changes in IOP (60). The proportions

of keratan sulfate and chondroitin sulfate can determine openness or compactness of proteoglycans, which in turn affects the water-binding characteristics of the tissue. A GAG chain with a higher concentration of keratan sulfate is more open and smaller than one containing higher concentrations of chondroitin sulfate (13, 73). Similar effects of glucocorticoids on TM cells and IOP have been reported by others (45, 52, 61, 92). Although the exact mechanism of steroid-induced ocular hypertension is unknown, it is postulated that the corticosteroids stabilize the lysosomal membranes which impede the liberation of the catabolic enzymes that normally degrade GAGs (32).

Hypotheses

Review of the literature elucidates the importance of GAGs in maintaining normal IOP. Isolation, characterization, and localization of GAGs from various stages of POAG in the Beagle is likely to gain new information on the possibility that changes in these carbohydrate moieties may permit a better understanding of the pathogenesis of glaucoma.

Numerous articles have been published on POAG in the Beagle (10, 11, 20, 34-36, 46, 47, 77, 89, 90). It is important to evaluate the changes in GAG moieties in a suitable animal model under conditions independent of drug therapy, surgical procedures, or mechanical intervention. The glaucomatous Beagle is the animal model which most

closely resembles POAG of humans and, therefore, is the animal species of choice for use in studies designed to evaluate the GAG moieties of the aqueous outflow pathways.

The major hypothesis for this dissertation is: the GAGs are localized in the TM and constitute the major resistance to aqueous outflow. Localization of GAGs will be accomplished by enzymatic procedures and microspectrophotometry image processing system (Zeiss SEM-IPS) whereas resistance to aqueous outflow and the role of the GAGs will be determined by enzyme degradation and perfusion procedures.

The secondary hypothesis for this dissertation is that GAGs, during the course of POAG, undergo biochemical changes which impairs aqueous humor outflow and increase IOP.

The main objectives of these studies are to locate, isolate and characterize the GAGs of the canine TM and to determine their role in aqueous humor dynamics in normal and glaucomatous Beagles.

MATERIALS AND METHODS

Experiment 1: Perfusion Study with Testicular Hyaluronidase

A total of sixteen normotensive (Beagles and mixed breeds) and three glaucomatous dogs were used in this study. Eight of the normotensive canines were perfused for 30 minutes and, to determine the maximum effects, the other eight for 60 minutes. Within each group, the eyes were randomly selected to receive 0, 25, 50 or 100 I.U. (International Units) of Bovine testicular hyaluronidase (Sigma Chemical, St. Louis, MO). Testicular hyaluronidase was chosen because of its activity on a number of GAGs. This enzyme catalyzes the hydrolysis of β 1-4 linkage between the N-acetyl-hexosamine and D-glucuronate residues in hyaluronic acid, chondroitin 4-, and chondroitin 6-sulfate (54, 100). Steady-state perfusion was maintained at a baseline pressure of 20 mmHg. The data was analyzed by a general linear model procedure (multivariate analysis of variance).

No preanesthetics were used. Sodium thiamylal, 4%, (Surital, Parke-Davis, Detroit, MI) was administered intravenously via the cephalic vein at 17.5 mg/kg to induce general anesthesia. The dogs were intubated and placed in dorsal recumbency. Halothane (Halocarbon Labs, Hackensack, NJ) and oxygen were used to maintain a anesthetic

plane with an approximate heart rate of 120/minutes. Blood pressure was monitored by direct catheterization of the femoral artery with polyethylene tubing connected to a heparinized saline filled Statham pressure transducer (Statham model P23ID, Gould-Statham Inc., Oxnard, CA) connected to a polygraph (Grass model 7D, Grass Inst., Quincy, MA).

The anterior chamber was cannulated with a 23-gauge needle at the limbus for perfusion. The needle was connected to a transducer as noted above. A stopcock was placed between the transducer and the delivery syringe and a graduated column (buret) of saline (to maintain IOP) was used in order to regulate the system (Figure 3). A saline column was used to calibrate the transducer and polygraph prior to each experiment. A delivery system was used to administer the four doses of hyaluronidase into the anterior chamber. The hyaluronidase was dissolved in 0.4 ml of Hanks balanced salt solution (Grand Island Biol. Co., New York, NY). A second 23-gauge needle was used to cannulate the limbus, approximately 40° from the first needle. This needle was connected to a syringe in order to aspirate 0.4 ml of aqueous humor (prior to the hyaluronidase injection) to maintain the IOP at 20 mmHg. The glaucomatous eyes were perfused at the IOP measured prior to anesthesia. Outflow was measured by determining the total number of microliters per minute of perfused saline.

Following the perfusion study, all eyes were perfused with a cold

solution of 2% glutaraldehyde in a 0.1M phosphate buffer (pH 7.0) for ten minutes. The eyes were enucleated and a dorsal (12 O'clock) scleral incision was made. The tissue was stored for 24 hours in the glutaraldehyde solution under refrigeration. After 24 hours, the eyes were sectioned to expose the TM. Sections were subsequently stained with colloidal iron for GAGs (colloidal iron staining method of Grierson I and Lee WR, 43) and examined by transmission electron microscopy using ultrathin sections (Philips 200, Philips, Holland). The colloidal iron staining method consisted of a 12% acetic acid wash, kept at room temperature for 90 minutes in colloidal iron stain (pH 1.2) and washed again with 12% acetic acid (six times).

Experiment 2: Isolation of GAGs from the Trabecular Meshwork, Sclera and Iris-Ciliary Body

Animals used in this study were eight normal and eight glaucomatous Beagles with POAG. The glaucomatous Beagles were divided into three groups: Late or advanced glaucoma, ages ranging from 60 to 82 months (6 eyes); moderate glaucoma, ages 25 to 59 months (6 eyes); and early stage of glaucoma, ages 6 to 24 months (4 eyes). The stage of glaucoma in each animal was determined by tonometry (measuring IOP), tonography (C-values or aqueous outflow facility), fundal examination and gonioscopy; the procedures were described in earlier articles (37, 38). Normal age-matched animals were used to compare

the three stages of the disease (3 to 4 eyes in each group).

Dissection of the Anterior Segment

The anterior segment tissues were divided into three separate components for analysis of GAGs: anterior sclera (i.e., sclera adjacent to the TM), iris-ciliary body and TM. The animals were sacrificed by intravenous injection of pentobarbital (Beuthanasia, Burns-Biotec Labs, Oakland, CA). The eyes were rapidly enucleated and all extraocular muscles, conjunctiva and orbital tissue were excised. Corneas were removed from the eyes at the limbal region. The posterior scleral tissue was removed near the ora serrata. The anterior segment was bisected and the lens and lens capsule were removed. Excess vitreous was removed with absorbent tissue paper (Kimberly-Clark, Roswell, GA). The anterior segment strip was placed on a dissection block which was composed of corkboard and was located in an ice bath at 4°C. The tissue was attached to the corkboard with dissection pins and placed under a dissection microscope (Nikon SMZ-10, Nikon, Inc., Garden City, NY).

Isolation of the TM followed a modified procedure of Knepper et al. (59, 64). The iris was elevated with a forceps in order to expose the iridocorneal angle and pectinate ligaments. Using a Beaver scalpel handle with a number 65 Beaver blade, the iris-ciliary body

was removed from the TM by placing the blade parallel to the iris, forming a 45° to 50° angle to the sclera. Stroke-like movements with the scalpel were used to dissect the trabecular tissue from the base of the iris-ciliary body. Once the iris-ciliary body was removed, the tissue block was rotated 180°, exposing the TM from the posterior aspect. The scalpel blade was placed at a 45° angle between the trabecular tissue and the sclera just under Descemet's membrane in order to remove any corneal tissue which remained. Again with stroke-like movements the TM was isolated (scraped) from the scleral tissue. Scleral sections were removed occasionally for histology in order to confirm proper dissection techniques. The dissected tissue was immediately immersed in liquid nitrogen and stored in an ultrafreezer (-70°C).

Isolation of Glycosaminoglycans

GAGs were isolated, with modifications, according to the methods described in earlier papers (5, 19, 64, 74, 82, 93). These microscale techniques permitted the TM from a single eye to be analyzed for GAG contents without pooling tissues from several eyes.

The tissue was homogenized (Brinkmann Inst. Polytron, Westburg, NY) in chloroform-methanol (2:1v/v) and remained in this solution for 12 hours (1 ml/10mg of tissue) in order to remove lipids from the tis-

sue. The tissue residue was dried in vacuo (vacuum desiccator) over P_2O_5 for 24 hours. Dry-defatted tissue was weighed and resuspended in 0.2 M sodium borate buffer, pH 7.8 (1.0 ml/25 mg dry-defatted tissue). A 0.4% solution of pronase B enzyme (for the degradation of proteins) in 0.2 M sodium borate was added to the suspension to provide a total of 1 mg of enzyme/100mg dry-defatted tissue. The enzyme was administered in two dosages, one half was given at the start of digestion and the other half after 12 hours. One-hundred μ l of 0.02 M $CaCl_2$ was added for every 2 ml of total solution. The tissue suspension was placed on a shaker bath at 50°C for 24 to 48 hours. Trichloroacetic acid (TCA) was added to the suspension (for precipitation of proteins) to obtain a final concentration of 5% TCA. Samples are placed on ice for 20 minutes and and centrifuged at 12,000 xg for 20 minutes (Sorvall RC-5, DuPont Co., Wilmington, DE). The precipitate was re-suspended in 5% TCA, placed on ice for 20 minutes and centrifuged (12,000 xg for 20 minutes). To the supernatant, 3 volumes of 5% potassium acetate in ethanol was added and the suspension was allowed to stand overnight at 4°C to precipitate GAGs. The suspension was centrifuged (12,000 xg, 20 minutes) and to the precipitate the following reagents (1ml of each) were added: ethanol, ethanol:ether, (1:1 v/v) and ether (ethyl) with centrifugation (same as above) between each step. Care was taken not to disturb the pellet during each of the ethanol-ether steps in order to reduce the loss of GAGs. Samples

were dried in a vacuum desiccator over P_2O_5 at room temperature. The precipitate was resuspended in 500 μ l of 50 mM sodium phosphate buffer containing 5 mM $MgCl_2$ (pH 7.4). To the suspension 50 μ l of a 0.1% solution of deoxyribonuclease I (70 units Calbiochem, Behring Diagnostics, San Diego, CA) and 50 μ l of 0.1% solution ribonuclease A (180 units, Calbiochem) were added in order to remove nucleic acid contaminants. Samples were incubated on a shaker bath at 37°C for 1 hour. Precipitation (TCA, potassium acetate-ethanol), centrifugation and ethanol-ether rinses were as above. Samples were dried in a vacuum desiccator at room temperature overnight. The GAG fractions were resuspended in 250 μ l of 75 mM NaCl solution and loaded onto a HPLC (high-pressure liquid chromatography) size exclusion column (0.75 X 600 mm Varian MicroPak TSK GEL PW 3000 columns in a series) which was equilibrated in a mobile phase of 0.1 M ammonium acetate - 7.5% ethanol. The HPLC system (Varian 5060 LC - Vista CDS-401, Palo Alto, CA) was used to remove oligosaccharides with a molecular weight less than 2000 and to separate GAGs from the glycopeptides. The flow rate was 1 ml/minute, fractions 10 to 16 ml were the GAG fractions (excluded volume) and fractions 17 to 30 ml (included volume) represented the glycoproteins. The recovery rate of GAGs from this process was 95%. GAG samples were lyophilized, resuspended in 750 μ l of 0.1 M ammonium acetate - 7.5% ethanol, transferred to 1.5 ml microcentrifuge tubes and re-lyophilized. Samples were resuspended

50 μ l of 0.02 M sodium acetate - 0.15 M NaCl buffer for cellulose acetate electrophoresis and enzyme degradation studies.

Zone Electrophoresis of GAGs

Glycosaminoglycan fractions from the TM, iris-ciliary body and sclera were analyzed by zone electrophoresis (18, 19, 64, 70). The NIH standards (provided by Dr. P. A. Knepper) and isolated tissue GAGs were applied to a cellulose acetate membrane in a electrophoresis cell (Beckman R-10 Microzone cell, Beckman Inst., Fullerton, CA). The running buffer in the electrophoresis cell was 0.1 M lithium chloride in 0.01 N HCl with a pH of 2.2; the ionic strength of the buffer was $I = 0.06$. Cellulose acetate membranes were stabilized by electrophoresis for 5 minutes (4.5 mA constant current). Electrophoresis of the standards and tissue GAGs were then run under a constant current of 4.5 mA at approximately 100 volts for 15 minutes using a Beckman R-120 power supply. After electrophoresis, the cellulose acetate membranes were stained with alcian blue (8GX, Polyscience, Warrington, PA), rinsed and cleared in a series of acetate acid, ethanol and anhydrous methanol solutions in order to alter the porous nature of the acetate membrane (64). The membranes were dried in a oven (45°C) and analyzed by densitometry.

All chemicals used in the biochemical analyses were of reagent grade quality.

Densitometry

Quantitative analysis of the alcian blue stained membranes was accomplished by densitometry procedures. The NIH reference GAGs and tissue GAGs were scanned with a Gelman ACD-15 (Gelman Inst., Ann Arbor, MI) densitometer; specifications were 0-2.0 optical density scale, 0.10 sensitivity, 0.5 mm X 3 mm slit beam and a wavelength of 611nm. Planimetry procedures were used to determine the total area of each peak.

Experiment 3: Enzymatic Degradation of Tissue GAGs

Reference and tissue GAGs from the TM, iris-ciliary body and sclera were subjected to enzymatic degradation according to the following procedures (21). Table 2 illustrates the specific activity of all enzymes used in this dissertation.

Hyaluronidase, prepared from Streptomyces hyalurolyticus (Miles Scientific, Lisle, IL); was used to degrade hyaluronic acid. The incubation buffer was a 0.02 M sodium acetate - 0.15 M NaCl (pH 5.0); it contained 1 unit of enzyme (76). The substrate concentration and volume depend on the isolated tissue GAG concentration. Reference and tissue GAGs, equivalent to 5 µg of dry-defatted tissue weight, were placed in microcentrifuge tubes containing the incubation buffer-enzyme solution. Enzyme digestion was stopped after 30 minutes

by placing the tubes in boiling water for two minutes. The enzyme was precipitated by adding TCA, equivalent to 10% of the incubation volume, and allowed to stand on ice for 15 minutes. The mixture was centrifuged (12,000 xg, 20 minutes) and the precipitate was washed with 5% TCA solution. The GAGs were precipitated within the supernatant by adding a 5% potassium acetate-ethanol solution (overnight at 4°C). The GAGs were centrifuged (12,000 xg, 20 minutes) and washed with ethanol, ethanol:ether (1:1 v/v) and ether with centrifugation between each step. The GAGs were dried in a vacuum desiccator and dissolved in a 0.075 M NaCl solution. An aliquot was removed for cellulose acetate electrophoresis (15).

Chondroitin ABC lyase, prepared from Proteus vulgaris (Miles Scientific, Lisle, IL), was used to degrade hyaluronic acid, chondroitin sulfate and dermatan sulfate. The incubation buffer was 0.05 M Tris-HCl, 0.059 M sodium acetate, 0.05 M NaCl and 0.01% Bovine serum albumin (pH 8.0). The enzyme concentration was 0.1 unit of chondroitin ABC lyase (64, 87). Enzyme and GAG substrates were incubated for 60 minutes and the degradation was stopped by placing the microcentrifuge tubes in boiling water for 2 minutes. Precipitation of the enzyme and reisolation of resistant GAGs followed the same procedure listed in the hyaluronidase enzyme procedure.

Heparitinase (Miles Scientific, Lisle, IL), which is prepared from Flavobacterium heparinum, was used in the degradation of heparan sulfate. The incubation buffer was 10 μ moles of sodium acetate per

100 μ l at a pH of 7.0 and 0.1 unit of heparitinase. Enzyme and GAG substrate were incubated for 4 hours. at 30°C. The enzymatic degradation and precipitation were the same procedures described for hyaluronidase.

Heparinase (Miles Scientific, Lisle, IL) which degrades heparin; was prepared from Flavobacterium heparinum. Enzyme solution and precipitation procedures were the same as for heparitinase (68).

Keratanase (endo- β -D galactosidase, Miles Scientific, Lisle, IL) was used to degrade keratan sulfate. The enzyme was prepared from Pseudomonas species and was placed in an enzyme buffer containing 0.1 unit of enzyme with 5 μ M Tris-HCl (pH 7.2). The solution was incubated for 4 hours at 37°C (75). The tubes containing the enzyme were placed in boiling H₂O for 2 minutes. Glycosaminoglycans were precipitated according to the procedures listed for hyaluronidase.

The GAG residues were identified by alcian blue stained cellulose acetate membranes and quantitated by the densitometry procedure described previously.

Experiment 4: Localization of GAGs in the Trabecular Meshwork by Histochemical Procedures

Tissue specimens from Beagles representing: early (2 eyes), moderate (4 eyes) and advanced glaucoma (4 eyes), along with age-matched normals (2 eyes per each group), were fixed in 4% paraform-

aldehyde (for 24 hours) with 0.1 M cacodylate buffer (pH 7.2). Cetylpyridinium chloride (CPC) and polyvinylpyrrolidone (PVP) were added (0.5% of total volume) to the fixative to prevent GAG loss from the tissue (personal communication from P. A. Knepper 1986). Tissue was dehydrated in ethanol solutions (80-100%) and allowed to stand overnight in chloroform. All tissue was subsequently embedded in paraffin and sectioned at 8 μ m. Slides were then deparaffinized with xylene and ethanol rinses.

Two enzymes, hyaluronate lyase and chondroitin ABC lyase, and a combination of these two enzymes were used to degrade the GAGs histochemically (27). The enzyme buffers and concentrations were the same as those used in experiment three. An O-ring was secured around the tissue sections and the appropriate enzyme volume and concentration was added. Sections were incubated for 3 hours at 37°C. After the incubation period, the enzyme was removed and the sections were stained with alcian blue, 8GX (1%), pH 2.6, for 12 hours. The tissue was dehydrated with ethanol (80-100%) and xylene, covered (mounted with Permount, Fisher Sci., Springfield, NJ), and analyzed qualitatively and quantitatively by a computer-aided microspectrophotometer-Zonax/image processing system (SEM-IPS, Kontron-Zeiss, West Germany). The Zonax and Kontron systems are illustrated schematically in Figure 4. Tissue sections were placed on the stage of a standard light microscope (Zeiss) with a 63 X oil immersion objective for microspectrophotometry. The spectrophotometer spot measured 0.8 μ m in

diameter with an area of $0.502\mu\text{m}^2$. The monochromator setting was 610 nm for maximum absorption of alcian blue staining. The signal was transmitted to a photomultiplier system (Hamamatsu system, with an spectral response between 185 to 930 nm) and analyzed with the Zonax computer system. Calibration of the microspectrophotometer (100% transmission) was established by determining the percent transmission of a clear region adjacent to each tissue section.

Descemet's membrane, trabecular beams, juxtacanalicular zone and anterior sclera were analyzed for the concentration of alcian blue dye. A minimum of 10 microspectrophotometer readings were made for each ocular component mentioned above. The mean percent transmission in each tissue indicated the average concentration of alcian blue stain. The control, enzyme buffers and enzyme (hyaluronate lyase, chondroitin ABC lyase, and the combination of the two enzymes) treated tissue were analyzed in serial sections. The treated tissue sections and controls were analyzed in replicates and the results were expressed as the mean percent transmission plus or minus the standard deviation. A t-test was used to analyze the difference between the controls and enzyme treatment.

The iridocorneal angle and adjacent tissue were analyzed with a spectrophotometer; the data was digitalized and displayed graphically using the Kontron-Zeiss SEM-IPS system. Tissue sections of the TM from normal and glaucomatous animals were analyzed and displayed as percent transmission, with color-enhanced images from controls, enzyme buffers and enzyme-treated groups.

RESULTS

Experiment 1: Perfusion Study with Testicular Hyaluronidase

Normotensive eyes, perfused with 25, 50 and 100 I. U. of hyaluronidase, had an increase in the perfusion rate ($\mu\text{l}/\text{minute}$) over 30 minutes when compared to the control eyes (Figure 5). During the first fifteen minutes the perfusion rates were higher in the 25 and 50 I. U. hyaluronidase, but with greater variations than in the perfusion rates of the second fifteen minutes (Figure 5). Variation was also evident when comparing the control eyes and those infused with 100 I. U. of hyaluronidase (Figure 6). Dosages of hyaluronidase (25 and 50 I. U.) caused a significant increase in the perfusion rate when compared to the control eye ($P < 0.02$). Those eyes perfused with 100 I. U. of hyaluronidase indicated a significant difference in the perfusion rate ($P < 0.05$) only during the second fifteen minutes of a 30-minute perfusion.

In the glaucomatous dog, no significant differences in perfusion rates ($P < 0.63$) were detected between the infused eyes (25 and 50 I. U. of hyaluronidase), and the control eyes (Figure 7). The perfusion rates with 100 I. U. of hyaluronidase were similar to those results recorded for the 50 I. U. All of the glaucomatous dogs were in the advanced stage of the disease.

The normotensive eyes perfused for 60 minutes with 25, 50 or 100

I. U. of hyaluronidase showed increases in perfusion rates over the control eyes (Figure 8). The maximum effect of the enzyme occurred within the first 30 minutes. All dosages of hyaluronidase were significantly different ($P < 0.01$) when compared to the perfusion rates of the control eyes.

Figure 9 illustrates the normal canine trabecular beam stained with colloidal iron. After exposure to 100 I. U. of hyaluronidase, no colloidal iron staining was detected in the trabecular beams (Figure 10). Similar results were obtained with 25 and 50 I. U. of hyaluronidase.

In the glaucomatous eyes only minor changes occurred in the colloidal iron staining pattern of trabecular cells of eyes infused with 25, 50, or 100 I. U. of hyaluronidase (Figures 11-12). This indicates a possible enzyme-resistant material in the TM of the advanced glaucoma eye which is not present in the normal canine eye.

Experiment 2: Isolation of GAGs from the Trabecular Meshwork, Sclera and Iris-Ciliary Body

Isolation of GAGs from the TM, sclera and iris-ciliary body were obtained from age-matched normal and glaucomatous eyes. Each animal was characterized by tonometry, tonography, fundus examination and gonioscopy (Table 3).

The dissected dry-defatted tissue weight isolated from the

sclera, iris-ciliary body and TM is summarized in Table 4. Normal sclera and iris-ciliary body dry-defatted weights did not differ substantially when compared to the early, moderate and advanced glaucoma. The size of the dissected TM from all animals were approximately the same, however, the dry defatted weight was different in the advanced glaucoma TM when compared to the other age groups.

The dissection techniques permits the isolation and identification of GAGs in the aqueous outflow pathway. Figure 13 is a sagittal section, through the iridocorneal angle of a normal canine, showing the corneoscleral TM, trabecular vein, uveal meshwork and the iris-ciliary body. After the cornea and the iris-ciliary body were removed, the TM appeared as a gelatinous pigmented strip. This material seemed to be more abundant in the advanced glaucomatous dog than in the moderate and early glaucomatous animals. The corneoscleral TM has been dissected away leaving the scleral and trabecular veins exposed (Figure 14).

Electrophoretic analyses of GAG standards and GAGs isolated from the TM are shown in Figures 15 to 18. The GAG profiles of normal dogs were hyaluronic acid, heparan sulfate and chondroitin-dermatan sulfates; this seems to be consistent throughout the various age groups. The early glaucomatous dogs had a GAG profile similar to that of the normal canine (Figure 16). As the disease progressed, however, the chondroitin-dermatan sulfates and heparan sulfate disappeared, leaving only hyaluronic acid and a unidentified alcian blue positive material

(Figure 17). In advanced glaucoma, the normal GAG profile disappears leaving the unidentified material as the major component of the TM (Figure 18).

The major GAG components of the iris-ciliary body were hyaluronic acid, chondroitin-dermatan sulfates and keratan sulfate in the normal canine, early and moderate glaucomatous dogs (Figures 19-20). The advanced glaucomatous dogs had a similar GAG profile except the chondroitin sulfates seemed to be more abundant than dermatan sulfate (Figure 21).

Following electrophoresis, the GAG profile from scleral tissue, was similar to the iris-ciliary body (Figure 19). Hyaluronic acid, chondroitin-dermatan sulfates and keratan sulfate were the major GAG components. This was evident for normal and glaucomatous eyes regardless of age, with the exception of two eyes. One advanced and one moderate glaucoma eye showed a banding pattern atypical of GAGs (Figures 22-23).

Experiment 3: Enzymatic Degradation of Tissue GAGs

Densitometry recordings of cellulose acetate membranes from early, moderate and advanced glaucomatous and from normal TM are shown in Figure 24. The isolated GAGs from the TM of normal and glaucomatous eyes were subjected to hyaluronate lyase enzyme degradation. In

normal and early glaucomatous TM, hyaluronate lyase reduced the alcian blue-stained band which co-migrated with standard hyaluronic acid (Figure 25). Hyaluronate lyase decreased the alcian blue-stained GAGs isolated from the TM of the moderate and advanced glaucomatous eyes, but the changes were not as completely degraded as normal TM GAGs (Figure 25).

Following enzyme degradation with chondroitin ABC lyase, heparitinase, heparinase and keratanase, all GAGs associated with the TM of normal and early glaucomatous eyes were removed (Figure 26). An enzyme-resistant material was identified in the TM of the moderate and advanced glaucomatous dogs which was not present in the normal and early glaucomatous eyes.

GAGs isolated from iris-ciliary body of early, moderate and advanced glaucoma and from normal eyes were subjected to hyaluronate lyase enzyme degradation. The enzyme reduced the band which comigrated with hyaluronic acid in the normal as well as in the glaucomatous eye (Figure 27). After degradation with chondroitin ABC lyase, heparitinase, heparinase and keratanase, all GAGs associated with the iris-ciliary body were removed (Figure 28).

Densitometry recordings of scleral GAGs from glaucoma and normal eyes are shown in Figure 29. Following enzyme degradation with hyaluronate lyase, chondroitin ABC lyase, keratanase, heparinase and heparitinase, all isolated scleral GAGs were removed (Figures 29-30). The two advanced glaucomatous eyes, which had atypical banding were

subjected to the GAG enzymatic procedures; all alcian blue-stained bands were removed.

Experiment 4: Localization of GAGs in the Trabecular
Meshwork by Histochemical Procedures

The alcian blue staining of Descemet's membrane, trabecular beams, juxtacanalicular zone and sclera (Figures 31-32) were measured with the microspectrophotometer (Zonax system). Table 5 shows the microspectrophotometric readings (expressed as percent transmission) for the early age normal and for early glaucomatous eyes. The enzyme buffer solutions did not change the percent transmission of alcian blue material when compared to the control sections. This was true for both normal and glaucomatous animals.

Hyaluronate lyase does not increase the percent transmission in most of the ocular sections, but chondroitin ABC lyase and the combination of the two enzymes (hyaluronate lyase and chondroitin ABC lyase) substantially increased the percent transmission. The early age normal and early glaucomatous eyes had similar microspectrophotometric readings when subjected to enzymatic degradation (Table 5).

Table 6 summarizes the microspectrophotometer readings from the moderate age normal and glaucomatous eyes. Recordings of alcian blue-staining patterns from Descemet's membrane were similar for controls as well as enzyme treated sections in the glaucomatous eyes.

In the normal eyes a increase in percent transmission was noted when comparing the control with the enzyme treated sections.

In the trabecular beams, juxtacanalicular zone, and sclera of the normal eye hyaluronate lyase caused an increase in the percent transmission. The chondroitin ABC lyase and combination of enzymes produced a substantial increase in the percent transmission when compared to the control and enzyme buffers. Similar results were seen in the trabecular beams, juxtacanalicular and sclera of the glaucomatous eye when comparing the controls and enzyme treatments (Table 6).

Comparing the trabecular beams, juxtacanalicular zone, Descemet's membrane, and sclera of the moderate glaucomatous eye and the age-matched normal, the change in percent transmission was significantly ($P < 0.05$) less in the glaucomatous eye after treatment with chondroitin ABC lyase (Table 6). Similar results were noted in the trabecular beams and sclera ($P < 0.01$) after treatment with hyaluronate lyase and chondroitin ABC lyase combinations (Table 6). In the advanced age normal, the trabecular beams, juxtacanalicular and sclera showed a similar pattern of increased percent transmission after enzyme degradation (Table 7). The advanced age glaucomatous eyes showed no significant change ($P > 0.05$) in percent transmission after being subjected to hyaluronate lyase, chondroitin ABC lyase, and the combination enzyme treatment (Table 7). This suggested the presence of an enzyme resistant material or a masking effect preventing alcian blue-staining.

In order to ascertain the amount of alcian blue staining, the TM tissue was displayed using the Kontron SEM-IPS system. Figures 33 and 34 (early age normal and early glaucomatous eyes) show the changes in alcian blue staining after the tissue sections were subjected to GAG degrading enzymes. In the moderate age normals, the change in alcian blue-staining is evident (Figure 35) with an increase in percent transmission; but in the moderate age glaucomatous eye, the change in percent transmission is not remarkable, indicating the presence of a GAG-resistant material (Figure 36). The advanced age normals exhibited the anticipated increase in percent transmission after enzyme degradation (Figure 37). In Figure 38, the advanced glaucoma shows a high percent of transmission before enzyme treatment and no substantial change in percent transmission after enzyme treatment.

DISCUSSION

Testicular hyaluronidase was effective in decreasing the resistance to aqueous outflow in the normal eye, indicating the importance of hyaluronic acid and some of the chondroitin sulfates in regulating aqueous outflow.

The variability in the perfusion rate may be due to a single infusion of the hyaluronidase, especially with the 100 I. U. of enzyme. In another study, the hyaluronidase was perfused over 30 minutes, causing a steady decrease in the aqueous outflow resistance (65). The sudden decrease in perfusion rate, after the injection of 100 I. U. of hyaluronidase, followed by a period of varied perfusion, is apparently due to the high level of hyaluronidase present in the anterior chamber. Perfusion of in vitro human globes with high molecular weight proteins markedly lowered the infusion rate (49, 51).

In advanced glaucomatous eyes the perfusion rates were not changed when 25, 50 or 100 I. U. of testicular hyaluronidase were infused into the anterior chamber. This suggests that the advanced glaucomatous eyes contains a material in the TM which is resistant to hyaluronidase degradation. Since testicular hyaluronidase degrades hyaluronic acid and some of the chondroitin sulfates, it is possible that the GAG moieties have changed forming conjugates that are resistant to enzymatic degradation.

It is apparent from the electron micrographs of normotensive eyes that colloidal iron stains the GAGs along the endothelial cell walls and basement membrane and in the collagen fiber core (Figure 9). Testicular hyaluronidase was effective in removing the GAGs (thus the absence of colloidal iron staining) from the TM of the normal canine eye. No colloidal iron stain was present in the TM of normal eyes after hyaluronidase degradation. The eyes of other species perfused with hyaluronidase have shown similar results (6, 4, 59).

The electron micrographs of the glaucomatous eyes revealed that the colloidal iron stain remains approximately the same on the endothelium cell, basement membrane and collagen fibers after hyaluronidase perfusion. This suggests that a GAG moiety is present which is resistant to enzyme degradation and may be responsible for the lack of change in perfusion rates of glaucomatous eyes. The collagen fibers were larger in diameter and more irregular in shape than those of the normal eye. Similar results were reported in a recent study where marked differences in collagen fiber size and shape (larger and irregular) were noted in the advanced glaucomatous animal (39). In Figure 11, a matrix material within the intertrabecular space was stained with colloidal iron. The presence of this material in the TM of the glaucomatous eye may be responsible for the increase in resistance to aqueous outflow.

In experiment, two the increase in TM tissue weight (of the advanced glaucomatous eye) may be associated with the accumulation of

the amorphous material, which seems to increase as the disease advances: An increase in TM tissue weight is seen in the advanced age normal but not to the extent of the glaucomatous TM. Human POAG patients have been shown to have fewer cells/unit tissue area than normals (34). There may be an association between the decrease in cell populations and the increase of amorphous material in the iridocorneal angle and the juxtacanalicular zone of the glaucomatous Beagle.

The electrophoretic analysis of isolated GAGs from the TM of the glaucomatous Beagle has been reported for the first time. A progressive change in the GAG moieties occurs as the disorder advances. The enzyme resistant material obtained by this analyses seems to be the same material which has been identified (with the perfusion study and colloidal iron procedure) within the intertrabecular spaces of the iridocorneal angle.

In the normal TM, three distinct alcian blue bands (hyaluronic acid, heparan sulfate and chondroitin-dermatan sulfate) were present. This is similar to the GAG content of the human TM (109). In contrast, the early glaucomatous TM had only two major bands (hyaluronic acid and chondroitin-dermatan sulfate) present. The relative percent (densitometry recordings) were also different when comparing the normal and early glaucomatous TM. This implies that the GAG moieties are beginning to change early in the course of the disease. As the disease progresses, the hyaluronic acid, which is usually

identified in the moderate glaucoma TM, seems to disappear from the GAG profile of the advanced glaucomatous TM, leaving only the unidentified material. It is within the early and moderate age groups that the disease process is changing from a normal state to the abnormal condition, indicated by the GAG profile of the early and moderate glaucomatous eye.

Glycosaminoglycans isolated from normal TM were degraded by enzymatic procedures, whereas the GAG fractions isolated from moderate and advanced glaucomatous TM were not. Hyaluronate lyase, chondroitin ABC lyase, heparitinase, heparinase and keratanase were not effective in removing all of the GAG material from the isolated fraction. This suggests the presence of a GAG-resistant material in the iridocorneal angle of the moderate and advanced glaucoma animal.

The distribution pattern of the iris-ciliary body GAGs was similar in the normal canine, in early and in moderate glaucomatous eyes; this seems to indicate that the iris-ciliary body is not involved in the pathogenesis of POAG in the Beagle. The isolated GAGs from the canine and rabbit iris-ciliary body were similar, with hyaluronic acid, chondroitin-dermatan sulfate and keratan sulfate being the major components (82).

Most of the scleral tissue from normal and glaucomatous Beagles had a similar GAG profile, except for one moderate and one advanced glaucomatous eye. Recently, collagen fiber organization was studied in the iridocorneal angle of the normal and glaucomatous dog (39).

The data indicated that collagen fibers in the advanced age glaucomatous dog decrease in numbers along with a variation in fiber thickness. Similar changes could occur in the sclera of the glaucomatous Beagle which would account for the change in the GAG profile of the one moderate and one advanced glaucomatous eye. The Beagle usually has a buphthalmic condition associated with the advanced glaucomatous eye. This also may influence the biochemical profile of the sclera by altering the collagen fibers which contain GAG cross-likages (9, 78).

Histochemical technique with a computer-aided microspectrophotometer provides an innovative approach to the localization of GAGs (90, 106, 109). Glycosaminoglycans were localized in the trabecular beams and juxtacanalicular zone, along with the GAG enzyme-resistant material. The major GAG components of the trabecular beams and the juxtacanalicular zone appeared to be chondroitin sulfates, as indicated by the shift in percent transmission with chondroitin ABC lyase enzyme. Hyaluronic acid represents a small fraction of the total GAG pool, since only smaller changes in the percent transmission occurred when using the hyaluronate lyase enzyme. In a recent study, GAG contents, in the TM of normal and of human POAG eyes were evaluated using the microspectrophotometer Kontron system (SEM-IPS). The results indicated that in the trabecular beams and the juxtacanalicular zone, hyaluronic acid was a minor component, chondroitin sulfates were the major components (109). This study also indicated

that the juxtacanalicular zone contained a twofold increase in a GAG enzyme-resistant material than in the control eyes. Similar results in the Beagle model reiterate the importance of the Beagle model for studying POAG.

The histochemical technique indicated that the moderate glaucomatous dog has a enzyme-resistant material. This was not the case in the advanced glaucomatous dog, where a high percent of transmission was noted before enzyme treatment and no apparent changes after enzyme treatment. It is possible that this enzyme-resistant material in the advanced glaucoma is less polyanionic, which would account for the decrease in alcian blue staining; or perhaps only the external part of the molecule has lost its polyanionic nature while the core remains polyanionic. Both of these possibilities seem unlikely, since the electrophoretic data indicates that the enzyme-resistant material is polyanionic. In another study it was noted that advanced glaucomatous eyes have a decrease in TM cellularity (3). This may account for the high percent of transmission in the advanced glaucomatous Beagle, since there are fewer cells and less alcian blue-positive material per 8 μ m section. It is also possible that a masking phenomenon exist in the advanced TM since proteins, nucleic acids and collagen remain in the tissue sections, crosslinkages may form preventing the penetration of the alcian blue stain.

Although age-related changes occur in the normal TM, the changes noted in this study appear to be related to the biochemical

alternation of normal metabolism. Alvarado, Floyd and Polansky (29, 79) have implicated the role of fibronectin in POAG, while Johnson (55, 56) has indicated that proteins in the aqueous humor may be involved in blocking the aqueous outflow. Rohen (86) noted in a recent study that thickening of the elastic-like fiber in the TM occurs with increasing age. This seems to have little influence on the aqueous outflow resistance in a normal eye. In a glaucomatous eye, the hypothesizes that a decreased thickness of the TM and shortening of the connecting fibrils will reduce the ability of the tissue to expand, thus decreasing the influence of the ciliary muscle tone on the outflow resistance. This may result in an under perfusion of a trabecular area thus increasing the amount of extracellular material (86). This may explain the mechanical mechanism involved in the disease process but it does not explain the biochemical differences that have been documented in this study and others. (32, 40, 59, 108).

Primary open angle glaucoma has also been postulated to be a form of pseudoexfoliation glaucoma (51) in which a compound (such as a glycoprotein or carbohydrate) is produced in the iris-ciliary body, is released into the aqueous humor and migrates to the TM matrix. This compound could bind to the GAG moieties, causing a decrease in aqueous outflow. Although the pseudoexfoliation theory seems to be an unlikely explanation for POAG, it has not been disproved.

The enzyme resistant material isolated from the moderate and ad-

vanced glaucoma TM needs to be analyzed to determine its molecular weight and chemical composition. Based on the isolation procedures and the preliminary results of assay procedures for uronic acids (modified Blumenkrantz assay) hexosamines and N-acetylated hexosamines (Elson-Morgan assay, 59, 60), the enzyme resistant material appears to be a GAG conjugate. Corticosteroids have been implicated in changing the metabolic function of lysosomal membranes, this in turn impedes the liberation of the catabolic enzymes that normally degrade GAGs (32). This process could account for the GAG conjugate which was isolated in the moderate and advanced glaucomatous dog; implicating a cytoskeletal problem. It is known that the assembly and processing of glycoproteins and GAGs occur within the endoplasmic reticulum (ER) and Golgi complex of a cell. The synthesis of GAGs also involves a series of glycosyl transferases and sulfotransferases which catalyze the transfer of a monosaccharide from the nucleotide sugar (UDP) to an appropriate acceptor within the ER and Golgi complex (102). It is conceivable that a malfunction in the biosynthesis of GAGs (within the endothelial cells of the TM) could account for this GAG conjugate found in the moderate and advanced glaucoma dog.

The perfusion study implicated the presence of an enzyme resistant material in the glaucomatous TM which was absent in the normal eye. Electron micrographs indicated the presence of colloidal iron positive material in the TM of the glaucomatous eye after exposure to hyaluronidase degradation. Biochemically the GAG profile

begins to change in the early stage of POAG, leaving an unidentified GAG material present in the TM of the moderate and advanced glaucomatous eye. Enzymatic degradation of tissue GAG revealed that the moderate and advanced glaucomatous eyes contained an enzyme-resistant material not found in the normal TM. This may implicate that the biosynthesis or degradation of the GAGs change during the course of the disease. The localization of GAGs in the TM by histochemical procedures revealed that the enzyme-resistant material is present in the trabecular beams and the juxtacanalicular zone of the TM. It can be concluded from these studies that the GAGs play an important role in normal aqueous outflow. During the course of POAG the GAG profile changes causing a blockage of aqueous outflow.

The data presented implies that POAG in the glaucomatous Beagle parallels the human condition, especially since an enzyme-resistant material was found in both the Beagle and human TM (40). This increases the value of the Beagle model for studying POAG, since both the human and Beagle TM may have a local defect which accumulates and enzyme resistant material over time. Future studies involving the early and moderate age glaucomatous eyes may reveal the possible pathogenesis of POAG which will benefit both human and animal.

SUMMARY

The perfusion study in combination with colloidal iron staining procedures, indicated that the Bovine testicular hyaluronidase, a GAG-degrading enzyme, increased the aqueous outflow in the normal canine eye, but not in the glaucomatous Beagle. Hence this study suggests that an enzyme-resistant material exists in the iridocorneal angle of the glaucomatous dog; this material is absent in normal dogs.

GAGs were isolated and analyzed biochemically from TM, iris-ciliary body and the sclera (adjacent to the iridocorneal angle) of normal and glaucomatous Beagles. The major GAG components of the normal and early glaucomatous TM were hyaluronic acid, heparan sulfate and chondroitin-dermatan sulfate; a GAG profile similar to that of human TM. In moderate and advanced stages of POAG in the Beagle, the profile consisted of hyaluronic acid and an unidentified GAG material. The unidentified material (which represented an enzyme-resistant GAG or a GAG conjugate) was the major component of the TM in the advanced POAG.

Tissue sections from normal dogs and glaucomatous Beagles were subjected to histochemical techniques and analyzed with a computer-aided microspectrophotometer and video image processing system (SEM-IPS). The data from this study indicated that an enzyme-resistant material is located in the trabecular beams and the juxtacanalicular zone of the glaucomatous eyes.

APPENDIX

TABLE 1: ANIMAL MODELS FOR GLAUCOMA COMPARED TO PRIMARY OPEN ANGLE GLAUCOMA IN HUMANS

| | PRIMARY OPEN-ANGLE GLAUCOMA - HUMAN | POAG GLAUCOMA BEAGLE | SPONTANEOUS GLAUCOMA (BUHLHAIMOS) - RABBIT | EXPERIMENTAL, ACUTE GLAUCOMA RABBIT AND NON-HUMAN PRIMATE |
|---|---|--|---|---|
| Inheritance | Dominant, incomplete penetrance or Auto- somal Recessive | Autosomal Recessive | Autosomal Recessive Semilethal Trait | None |
| Type of Glaucoma | Chronic Primary Open Angle Glaucoma | Chronic Primary Open Angle with Late Closure of Angle | Chronic Primary Open Angle with Coniodygenesis | Acute Iridocorneal Angle Obstruction |
| Postulated Pathogenesis | Possibly a biochemical, or functional defect of trabecular meshwork, Uveoscleral flow. | Possibly a biochemical or functional defect of trabecular meshwork or uveoscleral pathway | Physically defective out- flow pathway | Angle blocked after intra- cameral injection of foreign materials and associated inflammatory cells |
| Clinical Course | Progressive with perman- ent damage to intraocular structures | Progressive with perman- ent damage to intraocular structures | Progressive with perman- ent damage to intraocular structures | Variable duration few days to several weeks (esp. with repeated laser trabecular effects) |
| Intraocular Pressures (mmHg) | 22 (+2.5) N = 15.4 (+2.5) | 28.4 (+3.5) N = 21.4 (+2.1) | 21-50 N = 19.5 | Rabbit 20 - Primate 60 Primate N = 15 |
| Diurnal IOP Variation | yes | yes | yes | NA |
| Tonography (C-value) $\mu\text{l}^{-1}\text{mm}^{-1}\text{mmHg}$ | 0.18 (+2.5) (N = 0.28 (+0.5)) | 0.13 (+0.05) (N = 0.25 (+0.07)) | NA | NA |

TABLE 1: continued

| | PRIMARY OPEN-ANGLE GLAUCOMA - HUMAN | POAG GLAUCOMA BEAGLE | SPONTANEOUS GLAUCOMA (BUHHIHALMOS) - RABBIT | EXPERIMENTAL, ACUTE GLAUCOMA RABBIT NON-HUMAN PRIMATE |
|----------------------------------|--|---|--|---|
| Episcleral Venous Pressure | Normal and Affected 5-15 mmHg | Normal and Affected 10-12 mmHg | Normal and Affected 5-15 mmHg | NA NA |
| Conioscopy | Iridocorneal angle open throughout disease process | Angle open first 30- 32 months, narrow to closed angle in final stage (48 to 72 mos) | Angle open through disease. Pectinate ligaments absent or dysplastic, mesodermal sheets span angle | Angle not visible due to cells, foreign materials, inflammatory exudate. |
| Funduscopy | Cupping of optic disc with retinal vessel displacement | Cupping of optic disc with temporal demyelination at 18 months onwards | Severe excavation of normally cupped optic nerve head | Optic nerve cupping where visible through anterior chamber optics |
| Axoplasmic Flow | Reduced at scleral lamina cribrosa | Reduced at scleral lamina cribrosa | NA | Reduced |
| Blood Cortisol Level | Positive correlation with glaucoma | Positive correlation with glaucoma | NA | NA |
| % Total Flow Uveoscleral Flow | N = 4-14% | N = 15% Glaucoma = 3% | N = 13% | N = 30-65% (Nonhuman primate) |
| Pharmacologic Agents | Cholinergics CAI Epinephrine Timolol | Cholinergics CAI Epinephrine Timolol | CAI Timolol | Few Reports |

N = Normal

NA = Not Available

(Sources: 10, 11, 20, 34-36, 46, 47, 51, 77, 89, 90)

TABLE 2: SPECIFIC ACTIVITY OF ENZYMES

| ENZYME | SUBSTRATE SPECIFICITY | SPECIFIC ACTIVITY |
|-----------------------|---|---|
| Pronase | Most Proteins | 70,000 PUK (Proteolytic Unit/ Gram Dry Weight) |
| Deoxyribonuclease 1 | Nucleic Acid Contaminants in GAG Fractions | 2130 Kunitz Units/mg Dry Weight |
| Ribonuclease A | Nucleotide Contaminants in GAG Fractions | 90 Kunitz Units/mg Dry Weight |
| Hyaluronidase | Hyaluronic Acid | 2,000 TRU [*] /mg Protein |
| Chondroitin ABC Lyase | Hyaluronic Acid, Chondroitin 4(6-) Sulfate, Dermatan Sulfate | 1 Unit Liberates 1 Mole/ Min. at 37°C |
| Heparitinase | N-Acetyl, N-Sulfate Glucosyl Linkages of Heparan Sulfate | > 250 TRU/mg Protein |
| Heparinase | N-Sulfate and Glucosyl Linkages of Heparin | > 250 TRU/mg Protein |
| Keratanase | Keratan Sulfate | 1 Unit Liberates 1 Mole/ (Galactose)/1 Hour. |

*One TRU (Turbidity Reducing Unit) which causes 50% disease in O. D. at 660m/30 min. @ 60°C
(Source 64)

TABLE 3: PHYSIOLOGICAL DATA AND CLINICAL OBSERVATIONS ON THE GLAUCMATOUS BEAGLE AND NONDIENTENSIVE CANINE

| | ADVANCED GLAUCOMA | MODERATE GLAUCOMA | EARLY GLAUCOMA | NORMAL [*] |
|---|---|---|--|---------------------|
| Intraocular Pressure (mmHg) | 49 (\pm 21) | 29 (\pm 6) | 24 (\pm 3) | 21 (\pm 2) |
| Tonography ₁₋₁ (μ l min. ⁻¹ mmHg) | 0.07 (\pm 0.04) | 0.09 (\pm 0.06) | 0.20 (\pm 0.05) | 0.24 (\pm 0.07) |
| Gonioscopy | Narrowed to closed | Normal to narrow | Normal/Open | Normal/Open |
| Fundus | Optic atrophy pigmentation of optic disc. Retinal blood vessel attenuation | Central to paracentral cupping of the optic disc. Retinal blood vessels attenuation | Normal with some cupping of the optic disc | Normal |

From Experiment 2

S. D. = (\pm)^{*} Age-matched normals are reported as a single values since the range is narrow for all groups.

TABLE 4: ANTERIOR SEGMENT DRY DEFATTED TISSUE WEIGHTS FROM THE GLAUCOMATOUS AND AGE-MATCHED NORMAL EYES

| | ADVANCED GLAUCOMA | NORMAL | MODERATE GLAUCOMA | NORMAL | EARLY GLAUCOMA | NORMAL |
|---------------------|--------------------------------|-------------------|----------------------|-------------------|-------------------|-------------------|
| Trabecular Meshwork | 5.4 (± 2.5) [*] | 3.0 (± 2.4) | 1.7 (± 0.7) | 1.7 (± 0.5) | 1.0 (± 0.4) | 0.8 (± 0.6) |
| Iris-Ciliary Body | 58.2 (± 10) | 40.8 (± 7) | 53.0 (± 10) | 31.0 (± 9) | 32.9 (± 2) | 25.0 (± 6) |
| Sclera | 41.8 (± 5) | 42.0 (± 12) | 46.6 (± 11) | 54.0 (± 15) | 51.6 (± 2) | 43.3 (± 4) |

From Experiment 2

* Average weight in mg per eye \pm S. D.

TABLE 5: MICROSPPECTROPHOTOMETRY ANALYSIS OF EARLY AGE NORMAL AND GLAUCOMATOUS EYES

| TREATMENT | DESCOMET'S MEMBRANE | | TRABECULAR BEAMS | | JUXTACANALICULAR | | SOLERA | |
|---|---------------------|----------|------------------|----------|------------------|----------|--------|----------|
| | Normal | Glaucoma | Normal | Glaucoma | Normal | Glaucoma | Normal | Glaucoma |
| Control | 78 + 3 [*] | 82 + 2 | 66 + 3 | 59 + 2 | 64 + 3 | 62 + 2 | 80 + 3 | 73 + 2 |
| Hyaluronidase Buffer | 82 + 3 | 75 + 3 | 73 + 2 | 59 + 4 | 66 + 3 | 67 + 6 | 81 + 4 | 70 + 3 |
| Chondroitin ABC lyase Buffer | 73 + 1 | 72 + 3 | 65 + 6 | 64 + 3 | 59 + 3 | 60 + 3 | 76 + 3 | 74 + 2 |
| Hyaluronidase | 72 + 1 | 77 + 2 | 60 + 5 | 73 + 3 | 58 + 3 | 63 + 3 | 70 + 3 | 81 + 2 |
| Chondroitin ABC lyase | 86 + 2 | 89 + 2 | 94 + 2 | 92 + 2 | 97 + 2 | 98 + 5 | 98 + 2 | 99 + 2 |
| Hyaluronidase and Chondroitin ABC lyase | 82 + 2 | 84 + 2 | 85 + 2 | 84 + 2 | 90 + 3 | 92 + 3 | 96 + 2 | 99 + 1 |

From Experiment 4

* Expressed as mean percent transmission + S. D.

TABLE 6: MICROSPICHOIDOMETRY ANALYSIS OF MODERATE AGE NORMAL AND GLAUCOMATOUS EYES

| TREATMENT | DESCMET'S MEMBRANE | | TRABECULAR BEAMS | | JUNCTACANALICULAR | | SCLERA | |
|--|-------------------------|------------|------------------|---------------------------|-------------------|---------------------------|-------------|---------------------------|
| | Normal | Glaucoma | Normal | Glaucoma | Normal | Glaucoma | Normal | Glaucoma |
| Control | 83 \pm 2 [*] | 93 \pm 2 | 59 \pm 5 | 56 \pm 6 | 55 \pm 6 | 46 \pm 5 | 58 \pm 4 | 57 \pm 3 |
| Hyaluronidase Buffer | 84 \pm 2 | 92 \pm 2 | 57 \pm 4 | 62 \pm 5 | 57 \pm 4 | 52 \pm 3 | 62 \pm 3 | 68 \pm 5 |
| Chondroitin ABC lyase Buffer | 81 \pm 2 | 92 \pm 2 | 51 \pm 6 | 66 \pm 4 | 54 \pm 4 | 46 \pm 6 | 67 \pm 3 | 64 \pm 7 |
| Hyaluronidase | 88 \pm 2 | 95 \pm 2 | 63 \pm 8 | 73 \pm 6 | 60 \pm 4 | 61 \pm 5 | 72 \pm 4 | 61 \pm 5 |
| Chondroitin ABC lyase | 88 \pm 3 | 92 \pm 2 | 91 \pm 3 | 80 \pm 5 ^{**} | 95 \pm 3 | 86 \pm 6 ^{**} | 99 \pm 3 | 89 \pm 3 ^{**} |
| Hyaluronidase and Chondroitin ABC lyase | 88 \pm 1 | 93 \pm 6 | 92 \pm 5 | 75 \pm 7 ^{***} | 94 \pm 5 | 91 \pm 3 ^{***} | 100 \pm 1 | 88 \pm 6 ^{***} |

From Experiment 4

* Expressed as mean percent transmission \pm S. D.

** p < 0.05

*** p < 0.01

TABLE 7: MICROSPECTROPHOTOMETRY ANALYSIS OF ADVANCED AGE NORMAL AND GLAUCOMATOUS EYES

| TREATMENT | DESCMET'S MEMBRANE | | TRABECULAR BEAMS | | JUXTACANALICULAR | | SCLERA | |
|---|---------------------|----------|------------------|----------|------------------|----------|---------|----------|
| | Normal | Glaucoma | Normal | Glaucoma | Normal | Glaucoma | Normal | Glaucoma |
| Control | 92 + 3 [*] | 98 + 1 | 66 + 7 | 93 + 5 | 64 + 4 | 95 + 3 | 80 + 4 | 98 + 2 |
| Hyaluronidase Buffer | 93 + 3 | — | 52 + 6 | 98 + 2 | 58 + 4 | 99 + 1 | 78 + 4 | 99 + 1 |
| Chondroitin AEC lyase Buffer | 89 + 2 | 98 + 1 | 57 + 6 | 94 + 3 | 59 + 3 | 96 + 2 | 76 + 5 | 95 + 2 |
| Hyaluronidase | 86 + 3 | 99 + 1 | 57 + 5 | 91 + 4 | 61 + 4 | 95 + 2 | 72 + 2 | 93 + 2 |
| Chondroitin AEC lyase | 91 + 2 | 99 + 1 | 86 + 4 | 93 + 2 | 94 + 3 | 96 + 4 | 98 + 2 | 97 + 2 |
| Hyaluronidase and Chondroitin AEC lyase | 99 + 1 | — | 99 + 1 | 86 + 5 | 96 + 2 | 93 + 4 | 99 + .5 | 92 + 5 |

From Experiment 4

* Expressed as mean percent transmission \pm S. D.

— No Membrane Present

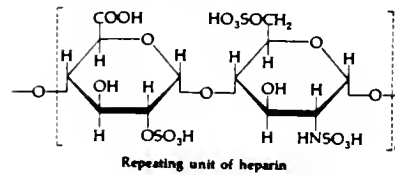
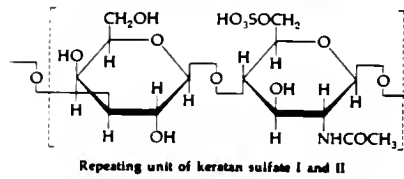
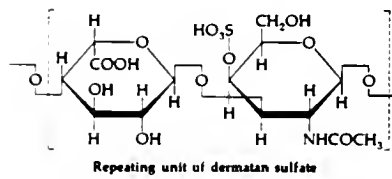
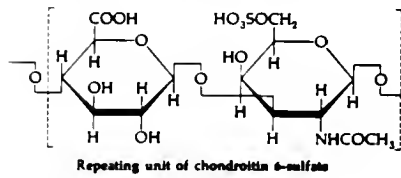
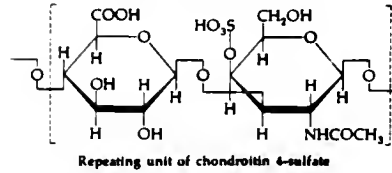
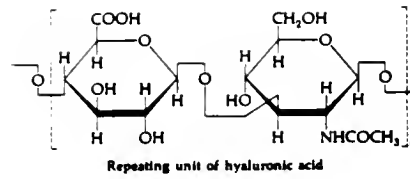


FIGURE 1. DISACCHARIDE REPEATING UNIT OF THE GLYCOSAMINOGLYCANS
(Source 102)

| NAME | COMPONENTS OF REPEATING DISACCHARIDE UNITS | LINKAGES AND SEQUENCE OF NITROGEN-CONTAINING GROUPS |
|--|---|---|
| Hyaluronic acid | D-Glucuronic acid (GlcUA), N-acetyl-D-glucosamine (GlcNAc) | ... GlcUA(β 1 \rightarrow 3)GlcNAc(β 1 \rightarrow 4)GlcUA(β 1 \rightarrow 3)GlcNAc(β 1 \rightarrow 4) ... |
| Chondroitin 4-sulfate (chondroitin sulfate A) | D-Glucuronic acid (GlcUA), N-acetyl-D-galactosamine 4-sulfate (GlcNAc-4S) | ... GlcUA(β 1 \rightarrow 3)GlcNAc-4S(β 1 \rightarrow 4)GlcUA(β 1 \rightarrow 3)GlcNAc-4S(β 1 \rightarrow 4) ... |
| Chondroitin 6-sulfate (chondroitin sulfate C) | D-Glucuronic acid (GlcUA), N-acetyl-D-galactosamine 6-sulfate (GlcNAc-6S) | ... GlcUA(β 1 \rightarrow 3)GlcNAc-6S(β 1 \rightarrow 4)GlcUA(β 1 \rightarrow 3)GlcNAc-6S(β 1 \rightarrow 4) ... |
| Dermatan sulfate* (chondroitin sulfate B) | L-Iduronic acid (IdUA), N-acetyl-D-galactosamine 4-sulfate (GalNAc-4S) | ... IdUA(β 1 \rightarrow 3)GalNAc-4S(β 1 \rightarrow 4)IdUA(β 1 \rightarrow 3)GalNAc-4S(β 1 \rightarrow 4) ... |
| Keratan sulfates I and II | D-Galactose (Gal), N-acetyl-D-glucosamine 6-sulfate (GlcNAc-6S) | ... Gal(β 1 \rightarrow 4)GlcNAc-6S(β 1 \rightarrow 3)Gal(β 1 \rightarrow 4)GlcNAc-6S(β 1 \rightarrow 3) ... |
| Heparan sulfate† and Heparin | D-Glucuronic acid 2-sulfate (GlcUA-2S), N-acetyl-D-glucosamine 6-sulfate (GlcNAc-6S) | ... IdUA-2S(β 1 \rightarrow 4)GlcNAc-6S(β 1 \rightarrow 4)GlcUA-2S(β 1 \rightarrow 4)GlcNAc-6S(β 1 \rightarrow 4) ... |

* Also may contain glucuronic acid.

† Also may contain N-sulfate derivatives of glucosamine rather than N-acetylglucosamine and variable amounts of iduronic and glucuronic acids.

FIGURE 2. COMPOSITION OF THE GLYCOSAMINOGLYCANS AND SEQUENCE OF THE LINKAGE REGION (Source 102).

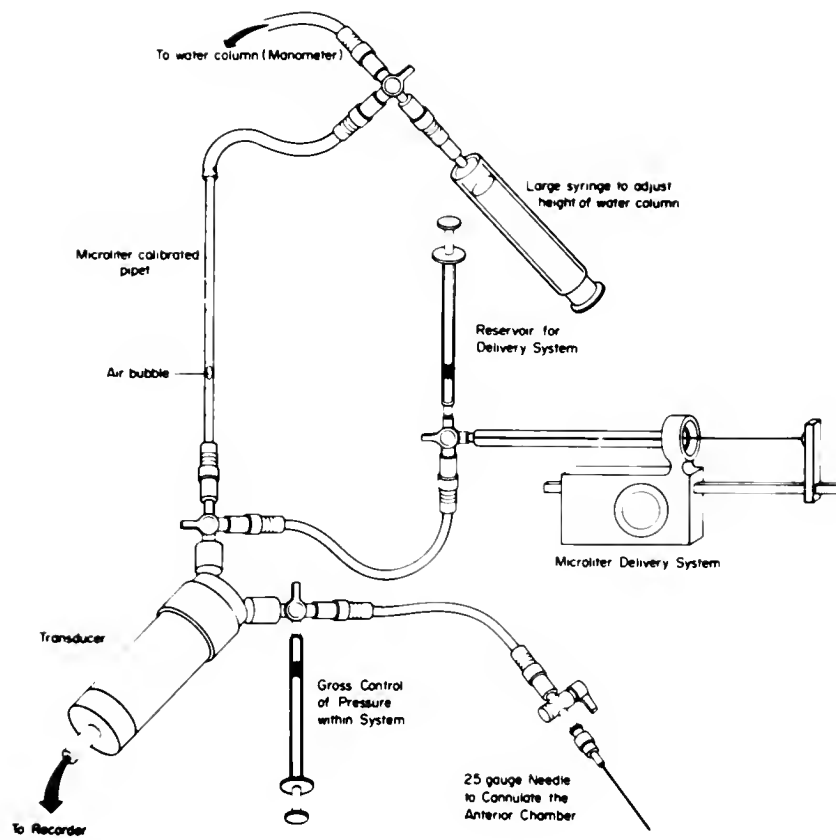


FIGURE 3. PERFUSION SYSTEM DIAGRAM (Source 77)

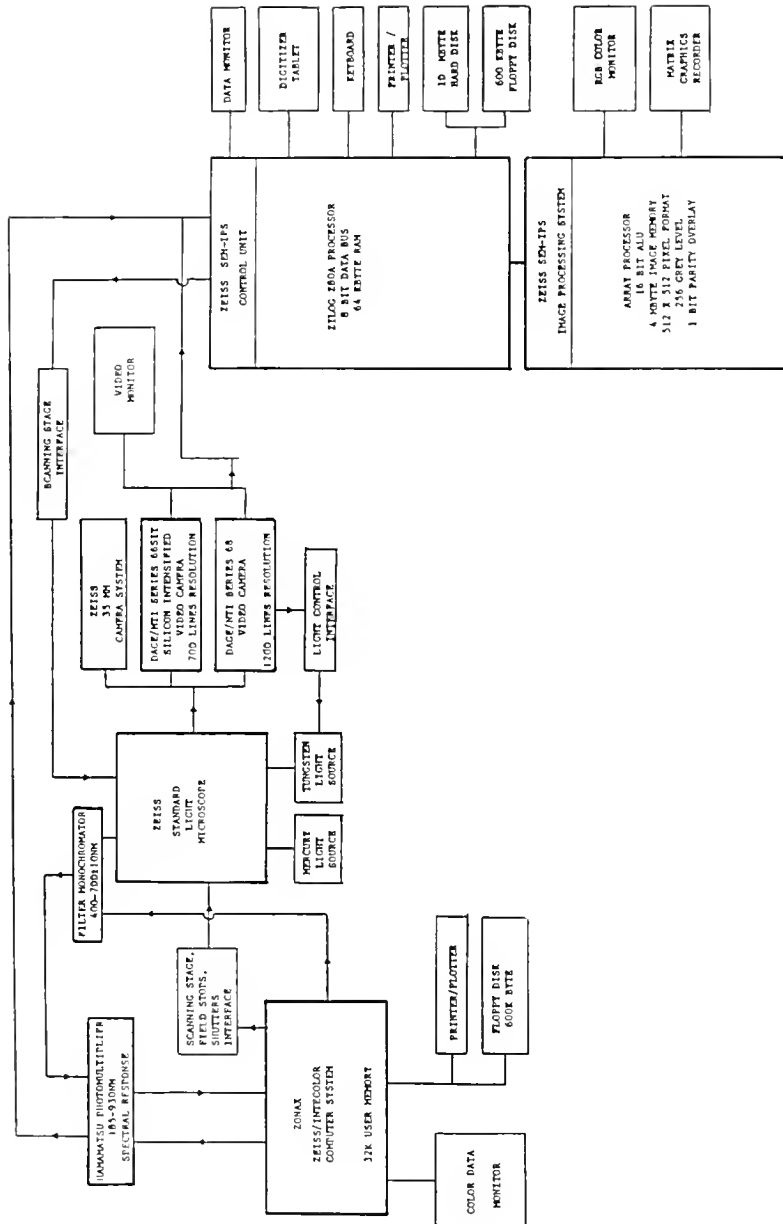


FIGURE 4. SCHEMATIC DIAGRAM OF THE ZEISS KONTRON SEM-IPS AND ZONAX SYSTEM.
(Source 40).

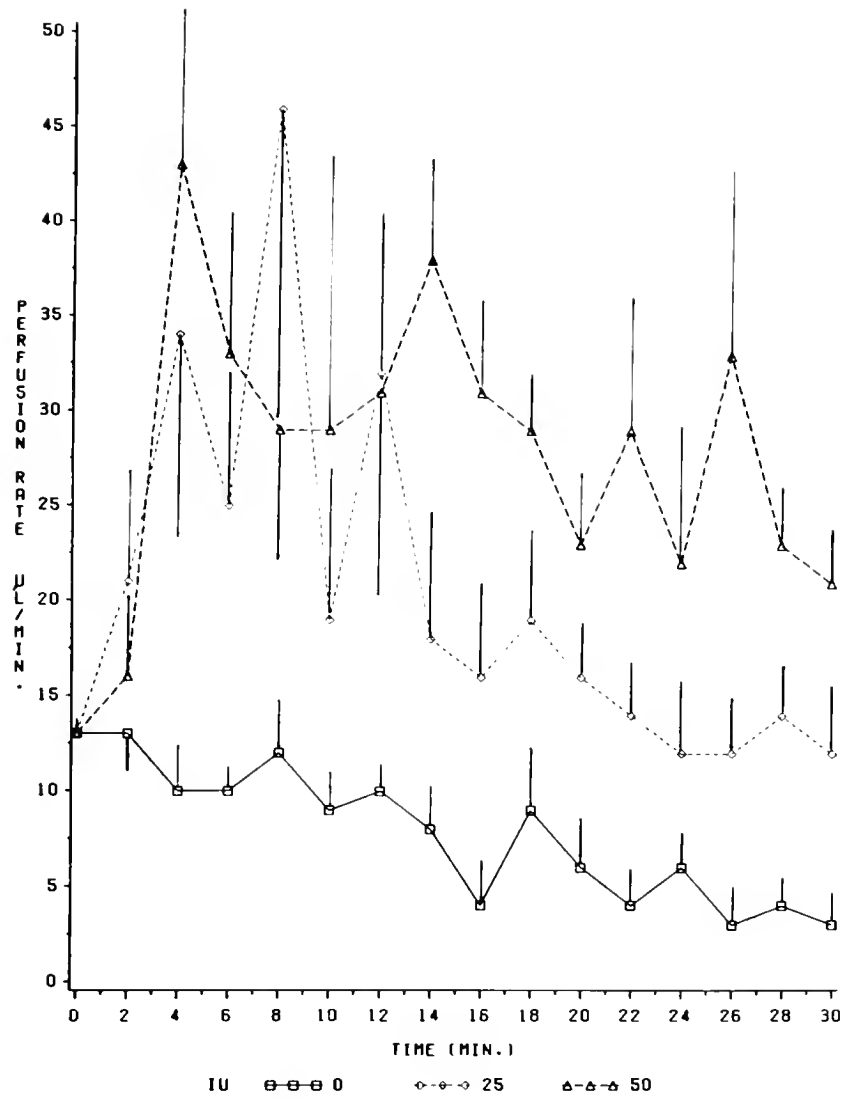


FIGURE 5. NORMAL CANINE EYES PERFUSED 30 MINUTES WITH 0, 25, AND 50 I. U. OF HYALURONIDASE. MEAN \pm STANDARD ERROR (S. E.)

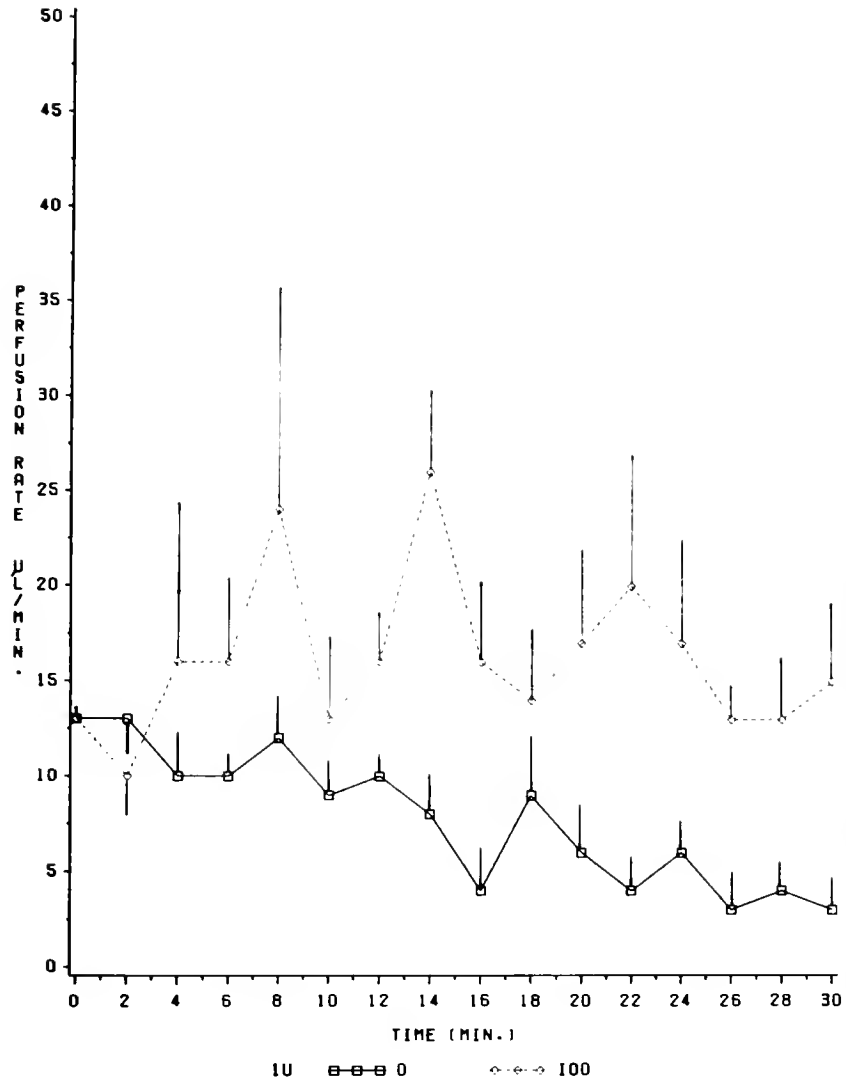


FIGURE 6. NORMAL CANINE EYES PERFUSED 30 MINUTES WITH 0 AND 100 I. U. OF HYALURONIDASE. MEAN \pm S. E.

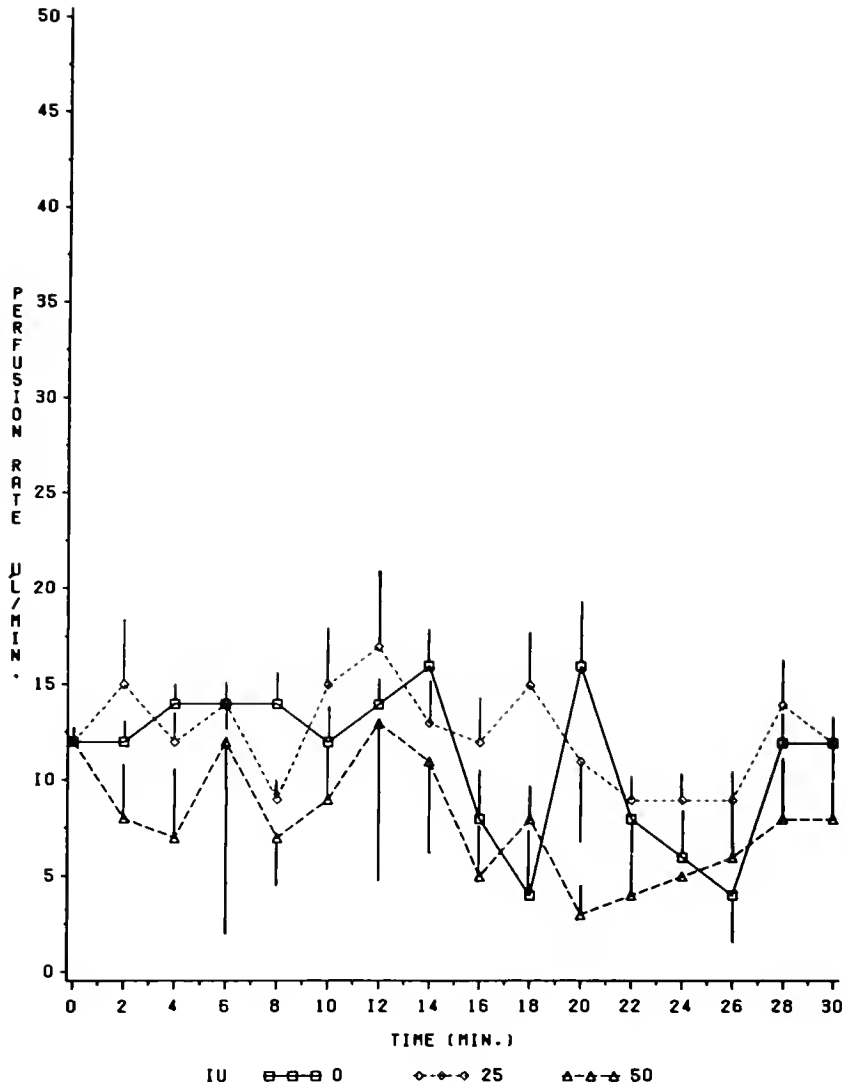


FIGURE 7. GLAUCOMATOUS BEAGLE EYES PERFUSED 30 MINUTES WITH 0, 25, AND 50 I. U. OF HYALURONIDASE. MEAN \pm S. E.

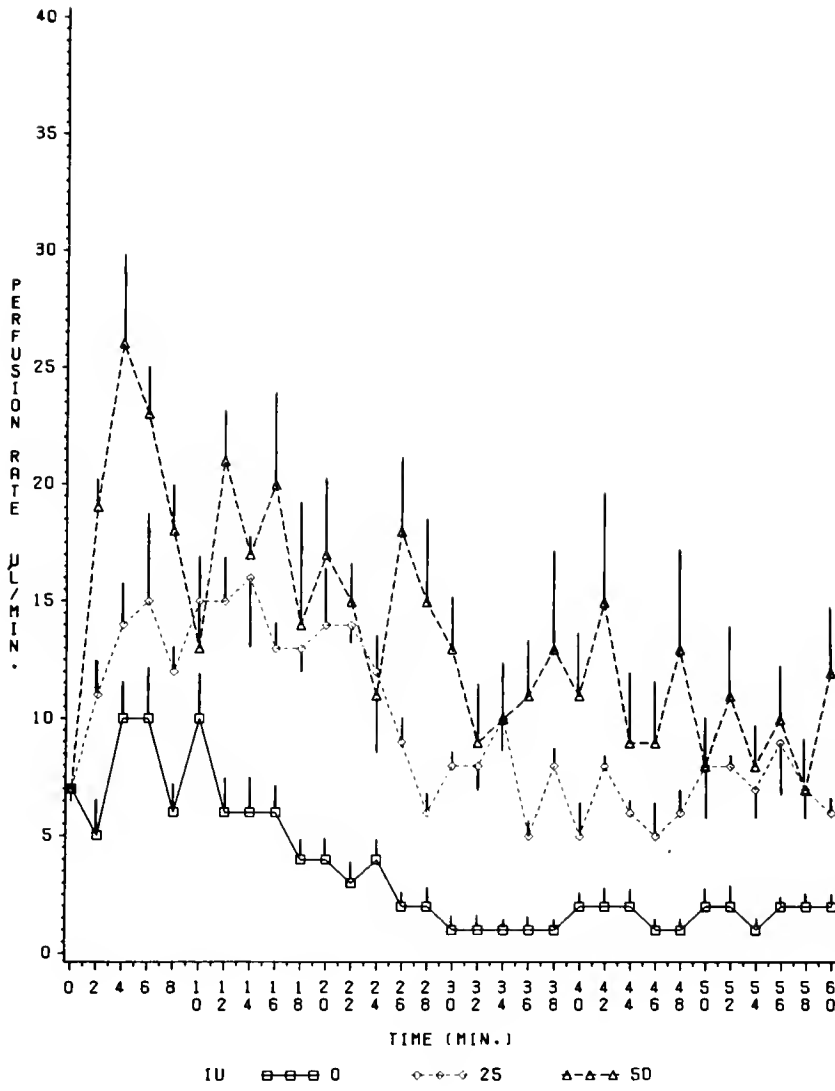


FIGURE 8. NORMAL CANINE EYES PERFUSED 60 MINUTES WITH 0, 25, 50 AND 100 I. U. OF HYALURONIDASE. MEAN \pm S. E.

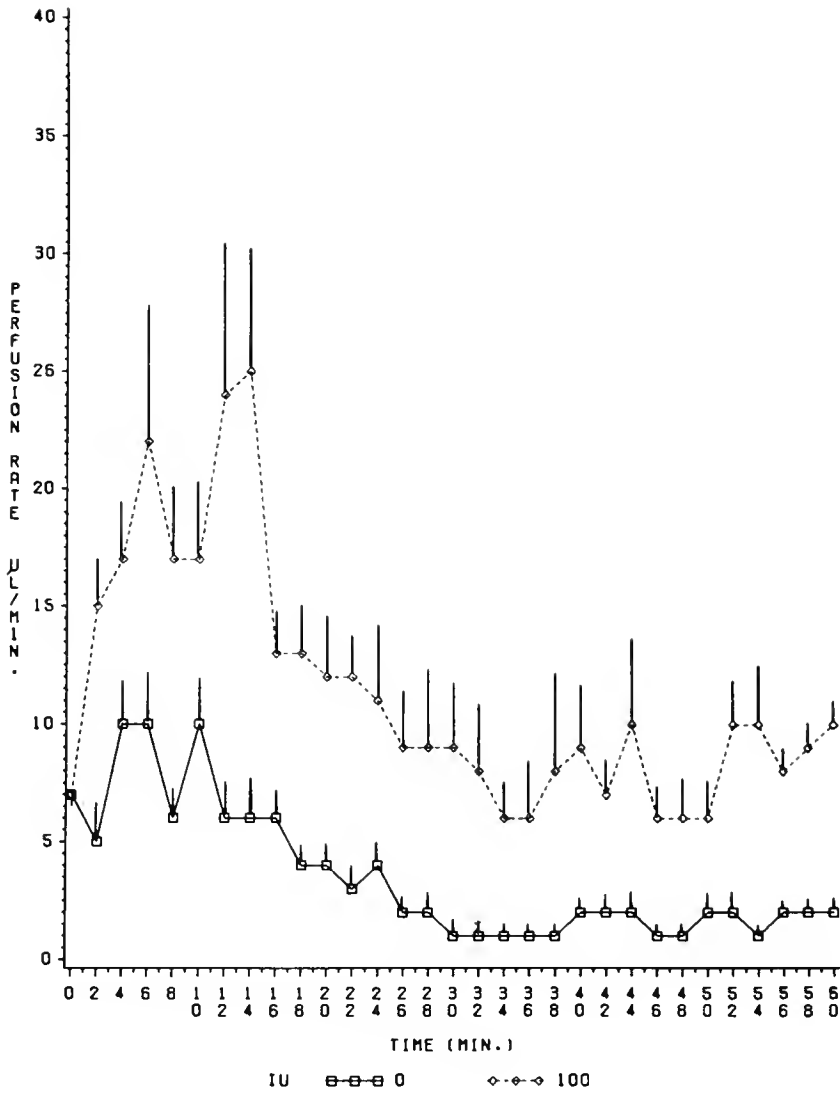


FIGURE 9. NORMAL CANINE EYES PERFUSED 60 MINUTES WITH 0 AND 100 I. U. OF HYALURONIDASE. MEAN \pm S. E.

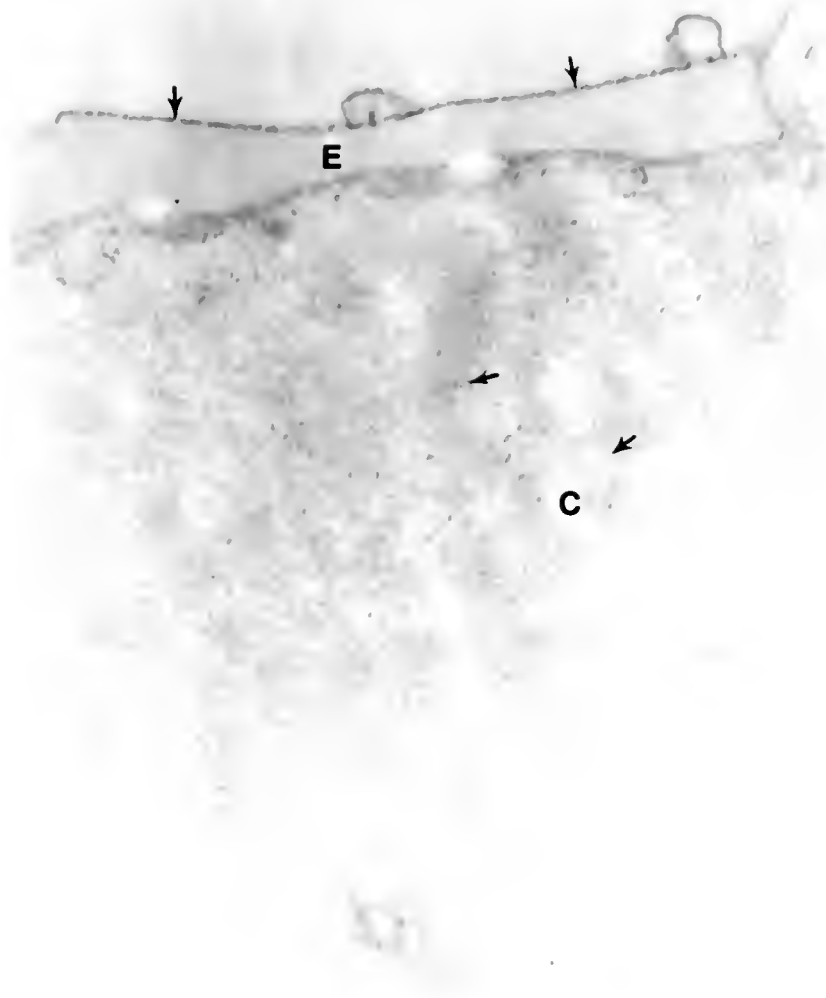


FIGURE 10. TRANSMISSION ELECTRON MICROGRAPH OF A NORMAL CANINE TRABECULAR BEAM, STAINED WITH COLLOIDAL IRON. Arrows = Colloidal iron stain, E = endothelial cell, C = collagen. (16,000 X).

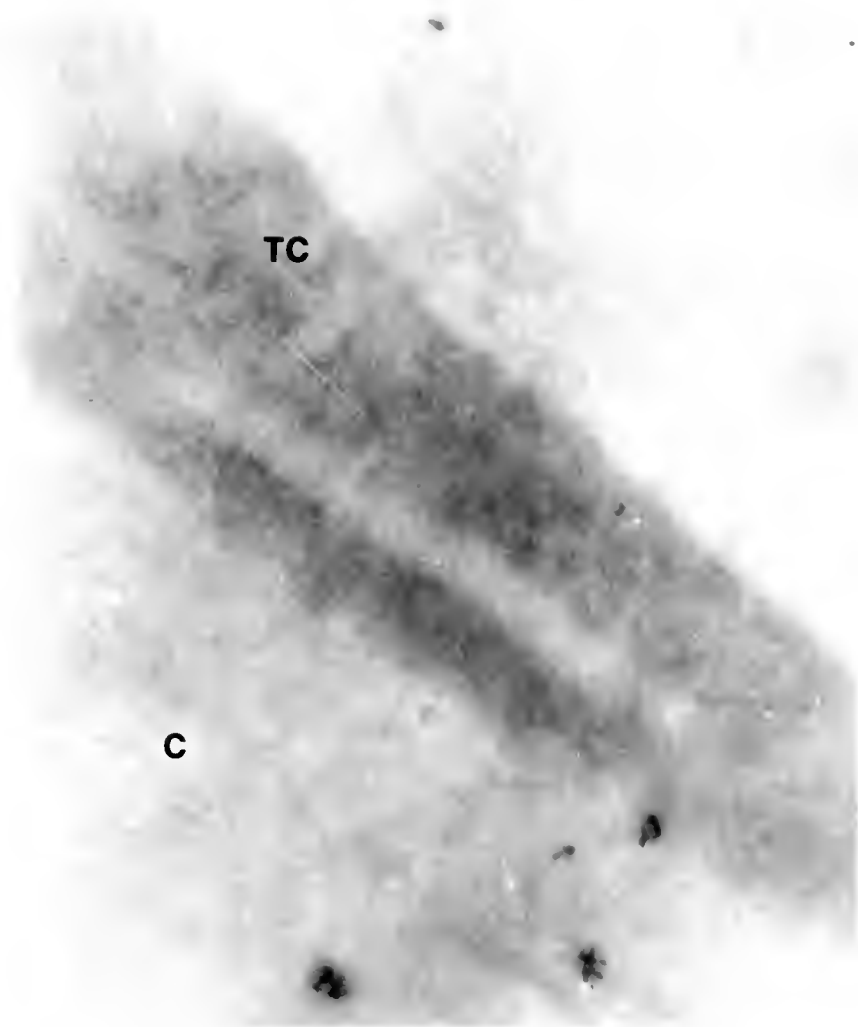
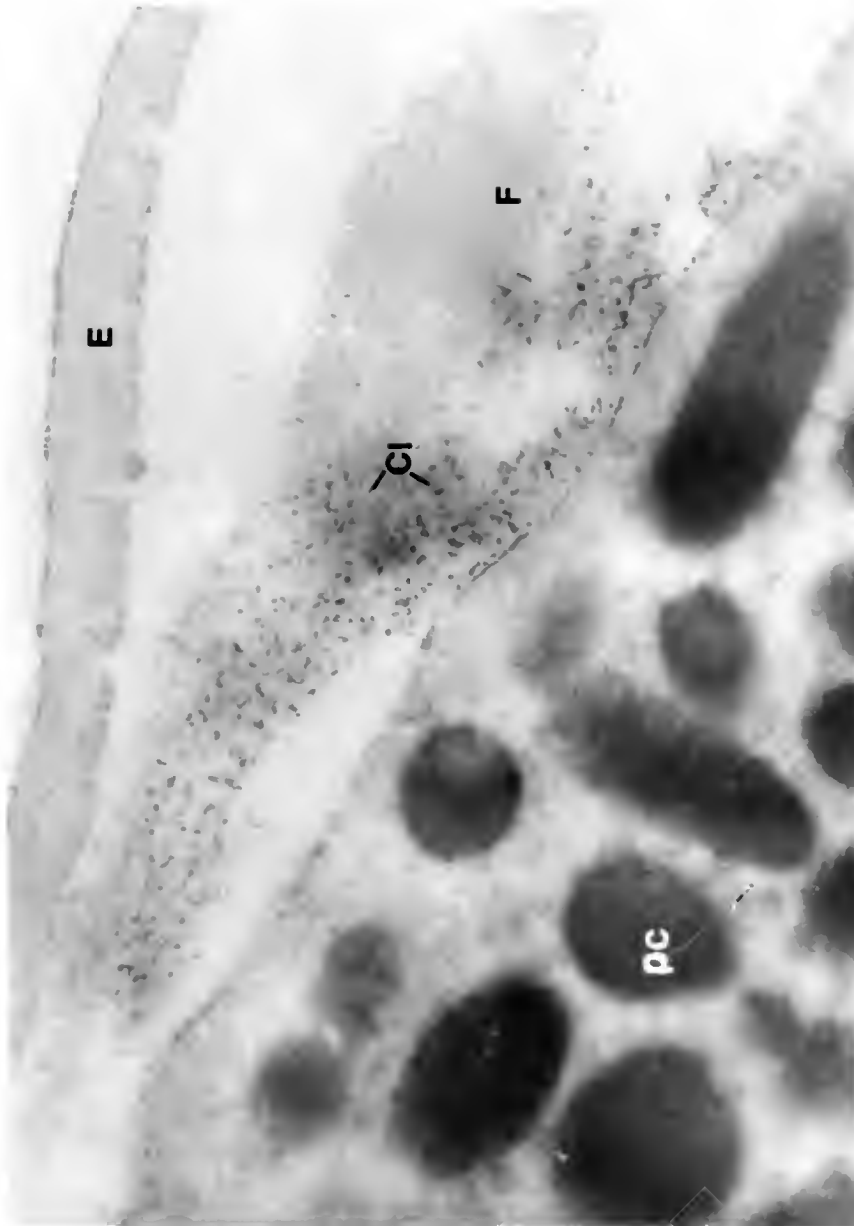


FIGURE 11. TRANSMISSION ELECTRON MICROGRAPH OF A NORMAL CANINE TRABECULAR BEAM, PERFUSED FOR 60 MINUTES WITH 100 I. U. OF HYALURONIDASE. TC = Trabecular cell, C = collagen. (25,000 X).



FIGURES 12. TRANSMISSION ELECTRON MICROGRAPH OF A TRABECULAR BEAM FROM AN ADVANCED GLAUCOMATOUS EYE, STAINED WITH COLLOIDAL IRON (arrows). E = endothelial cell, F = fibrillar material, CT = colloidal iron positive material within the trabecular spaces, PC = pigment cell. (26,000 X).

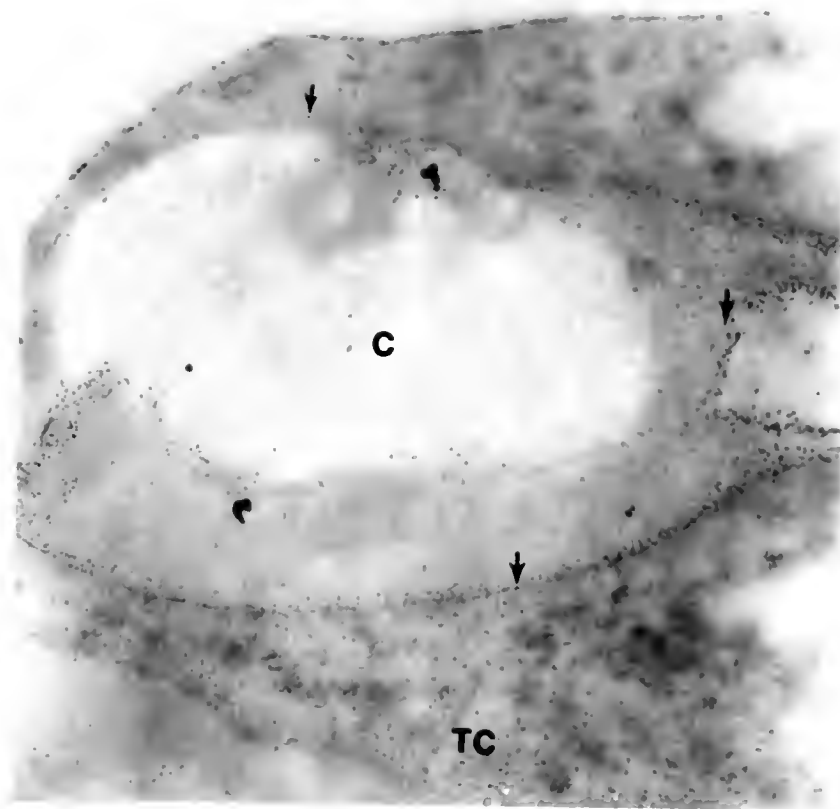


FIGURE 13. TRANSMISSION ELECTRON MICROGRAPH OF A TRABECULAR BEAM FROM AN ADVANCED GLAUCOMATOUS EYE, PERFUSED FOR 30 MINUTES WITH 100 I. U. OF HYALURONIDASE. Colloidal iron stain still present (arrows). TC = trabecular cell, C = collagen. (24,000 X).



FIGURE 14. NORMAL CANINE ANGLE. CM = corneal scleral trabecular meshwork, UM = uveoscleral meshwork, arrow = trabecular veins. (20 X) H & E. (Source 89)

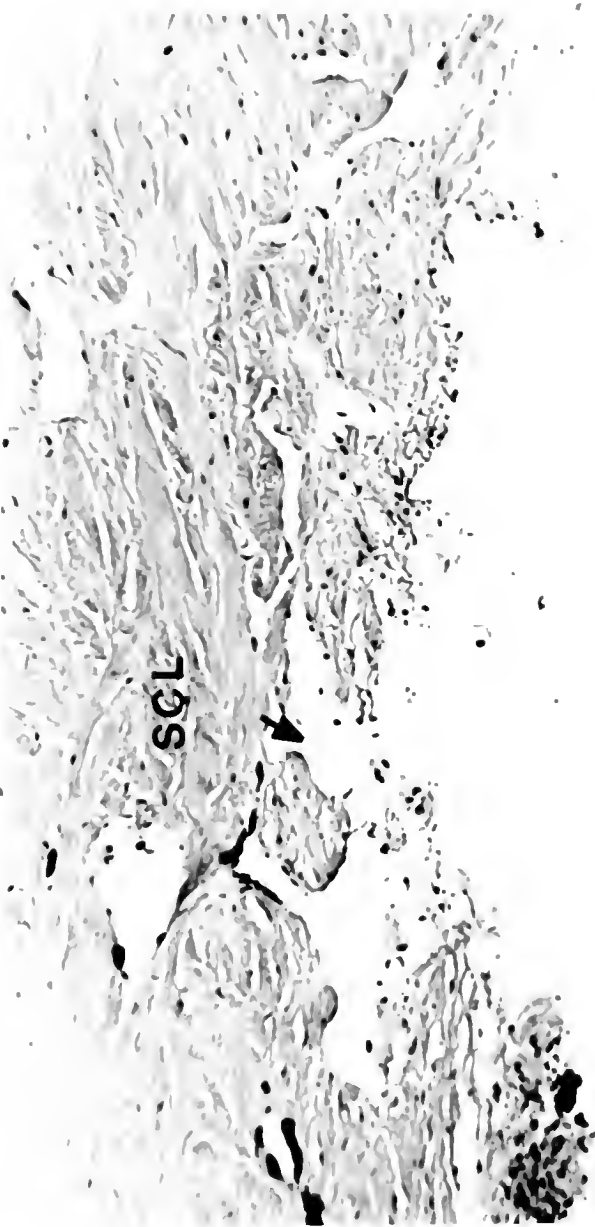


FIGURE 15. MICROGRAPH OF A SAGITTAL SECTION OF THE SCLERA WITH THE TRABECULAR MESHWORK REMOVED. SCL = sclera, arrow = trabecular veins. (40 X) H & E.

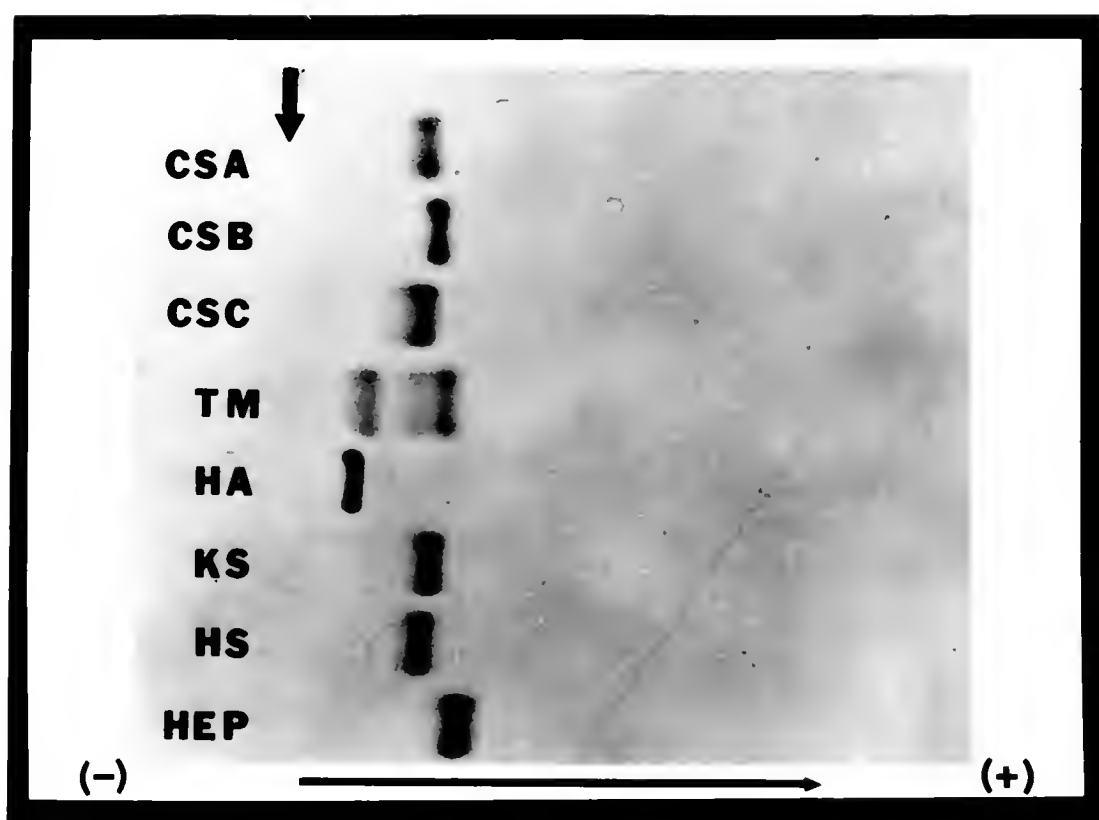


FIGURE 16. CELLULOSE ACETATE ELECTROPHORESIS OF NORMAL TRABECULAR MESHWORK. Aliquot of STD = $0.5 \mu\text{g}$ and TM = $0.5 \mu\text{g}$ in 0.1 M LiCl , 4.5 mA for 15 min . Abbr: CSA, CSB, CSC = Chondroitin A, B, C, TM = trabecular M., HA = hyaluronic acid, KS = keratan sulfate, HS = heparan sulfate, Hep = heparin, Sm. arrow = origin.

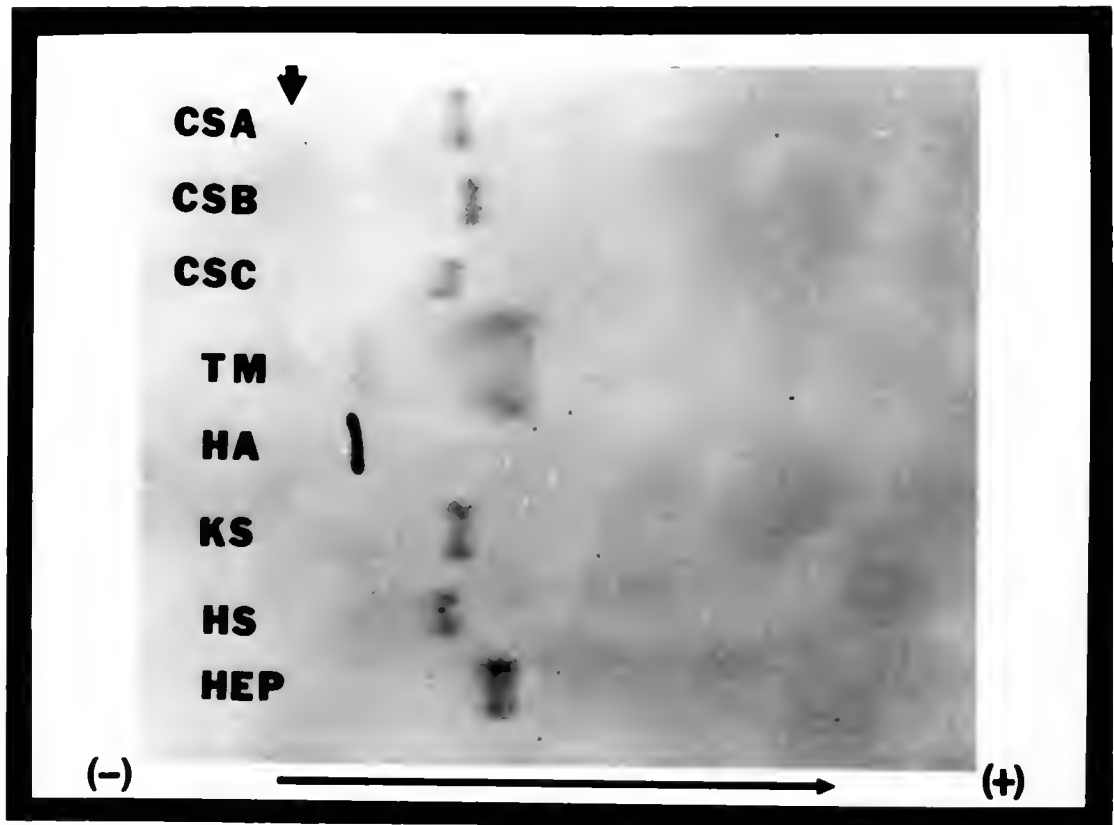


FIGURE 17. CELLULOSE ACETATE ELECTROPHORESIS OF EARLY GLAUCOMATOUS TRABECULAR MESHWORK. Aliquot of STD = 0.5 μ g and TM = 0.5 μ g in 0.1 M LiCl, 4.5 mA for 15 min. Abbr: CSA, CSB, CSC = Chondroitin A, B, C, TM = trabecular M., HA = hyaluronic acid, KS = keratan sulfate, HS = heparan sulfate, Hep = heparin, Sm. arrow = origin.

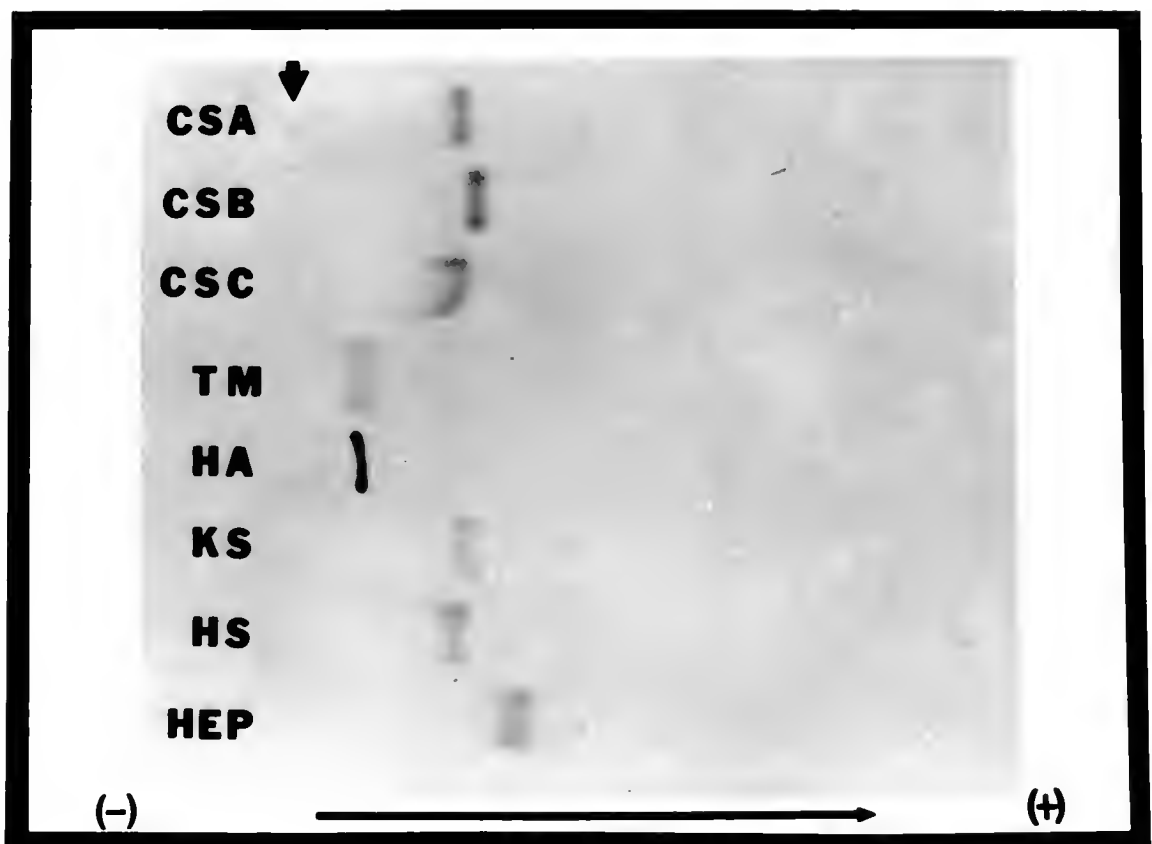


FIGURE 18. CELLULOSE ACETATE ELECTROPHORESIS OF MODERATE GLAUCOMATOUS TRABECULAR MESHWORK. Aliquot of STD = $0.5 \mu\text{g}$ and TM = $0.5 \mu\text{g}$ in 0.1 M LiCl , 4.5 mA for 15 min . Abbr: CSA, CSB, CSC = Chondroitin A, B, C, TM = trabecular M., HA = hyaluronic acid, KS = keratan sulfate, HS = heparan sulfate, Hep = heparin, Sm. arrow = origin.

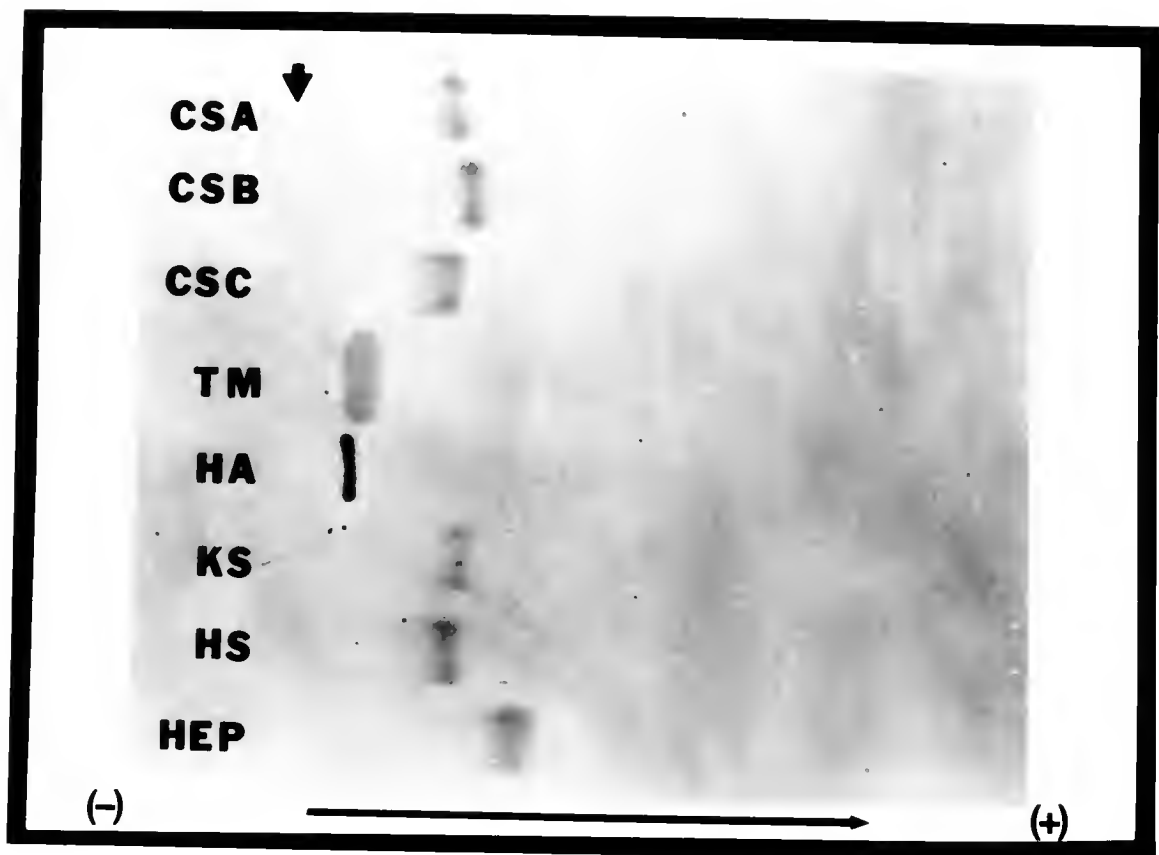


FIGURE 19. CELLULOSE ACETATE ELECTROPHORESIS OF ADVANCED GLAUCOMA-TOUS TRABECULAR MESHWORK. Aliquot of STD = $0.5 \mu\text{g}$ and TM = $0.5 \mu\text{g}$ in 0.1 M LiCl , 4.5 mA for 15 min . Abbr: CSA, CSB, CSC = Chondroitin A, B, C, TM = trabecular M., HA = hyaluronic acid, KS = keratan sulfate, HS = heparan sulfate, Hep = heparin, Sm arrow = origin.

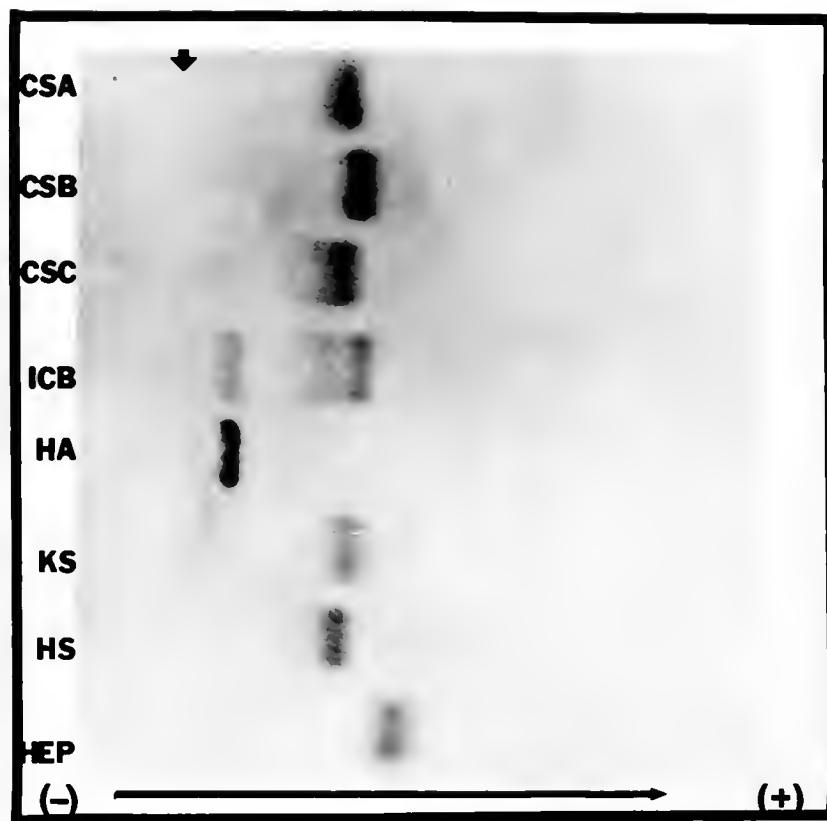


FIGURE 20. CELLULOSE ACETATE ELECTROPHORESIS OF NORMAL IRIS-CILIARY BODY. Aliquot of STD = $0.5 \mu\text{g}$ and IBC = $0.5 \mu\text{g}$ in 0.1 M LiCl , 4.5 mA for 15 min . Abbr: CSA, CSB, CSC = Chondroitin A, B, C, IBC = iris-ciliary body, HA = hyaluronic acid, KS = keratan sulfate, HS = heparan sulfate, Hep = heparin, Sm arrow = origin.

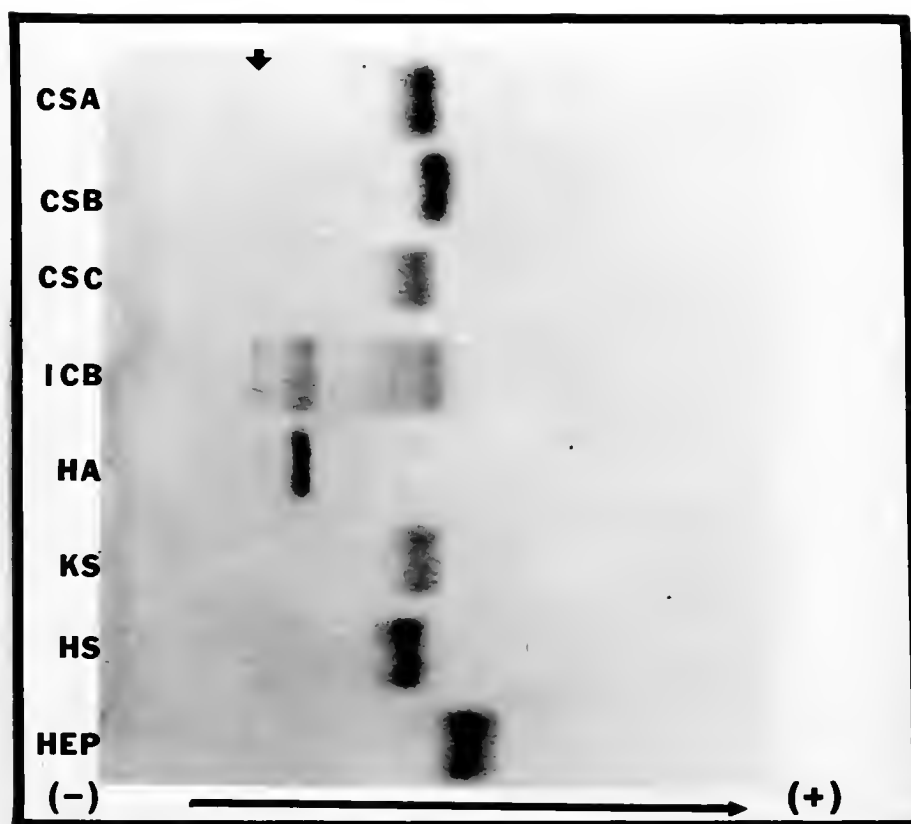


FIGURE 21. CELLULOSE ACETATE ELECTROPHORESIS OF EARLY AND MODERATE GLAUCOMATOUS IRIS-CILIARY BODY. Aliquot of STD = $0.5 \mu\text{g}$ and IBC = $0.5 \mu\text{g}$ in 0.1 M LiCl , 4.5 mA for 15 min . Abbr: CSA, CSB, CSC = Chondroitin A, B, C, IBC = iris-ciliary body, HA = hyaluronic acid, KS = keratan sulfate, HS = heparan sulfate, Hep = heparin, Sm arrow = origin.

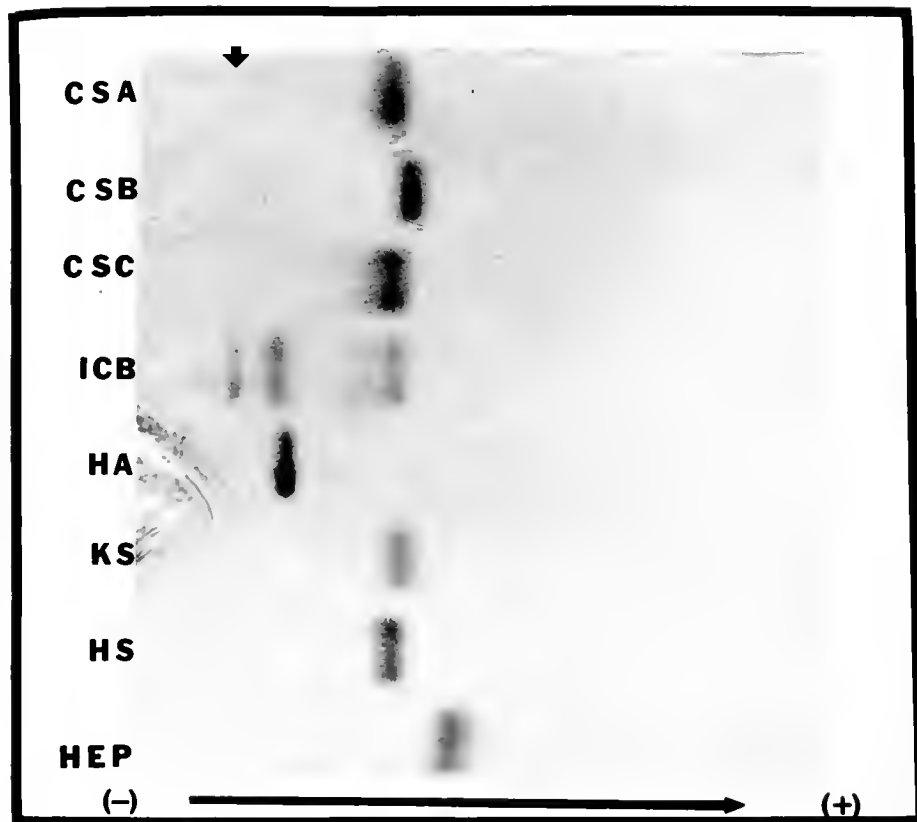


FIGURE 22. CELLULOSE ACETATE ELECTROPHORESIS OF ADVANCED GLAUCOMATOUS IRIS-CILIARY BODY. Aliquot of STD = $0.5 \mu\text{g}$ and IBC = $0.5 \mu\text{g}$ in 0.1 M LiCl , 4.5 mA for 15 min . Abbr: CSA, CSB, CSC = Chondroitin A, B, C, IBC = iris-ciliary body, HA = hyaluronic acid, KS = keratan sulfate, HS = heparan sulfate, Hep = heparin, Sm arrow = origin.

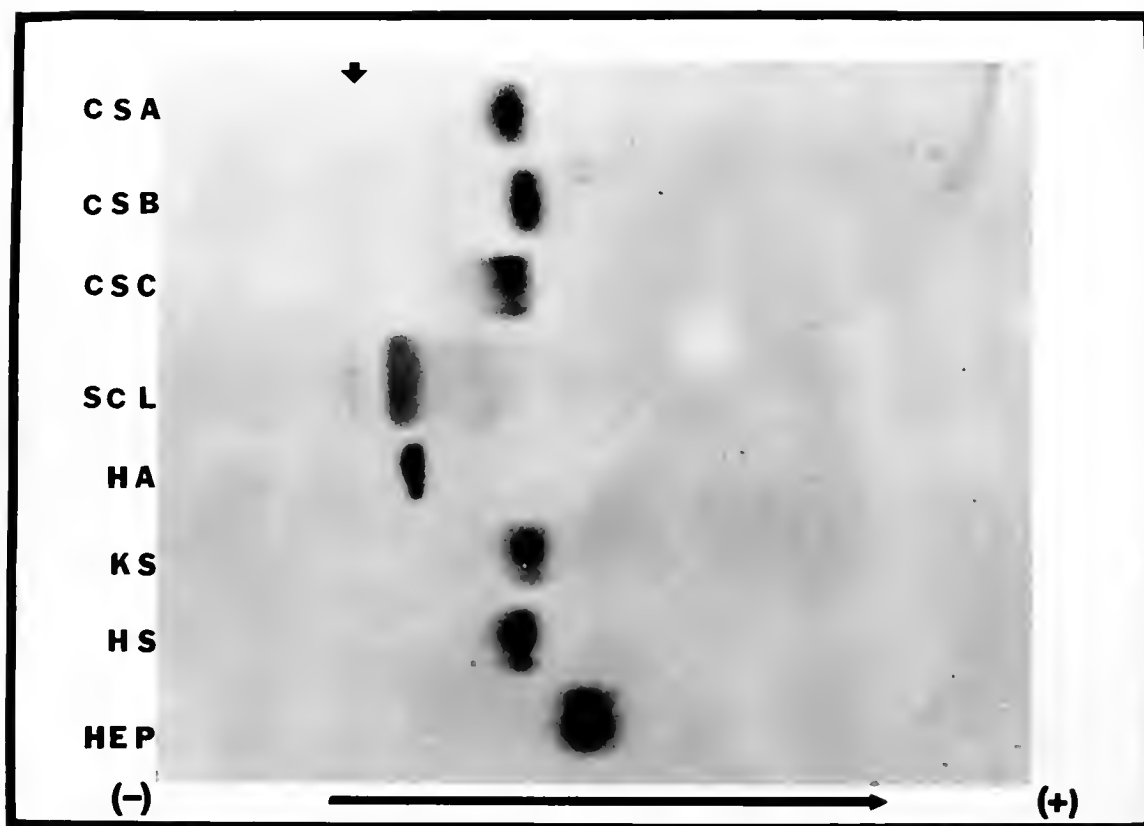


FIGURE 23. CELLULOSE ACETATE ELECTROPHORESIS OF ADVANCED GLAUCOMATOUS SCLERA. Aliquot of STD = $0.5 \mu\text{g}$ and SCL = $0.5 \mu\text{g}$ in 0.1 M LiCl , 4.5 mA for 15 min . Abbr: CSA, CSB, CSC = Chondroitin A, B, C, SCL = sclera, HA = hyaluronic acid, KS = keratan sulfate, HS = heparan sulfate, Hep = heparin, Sm arrow = origin.

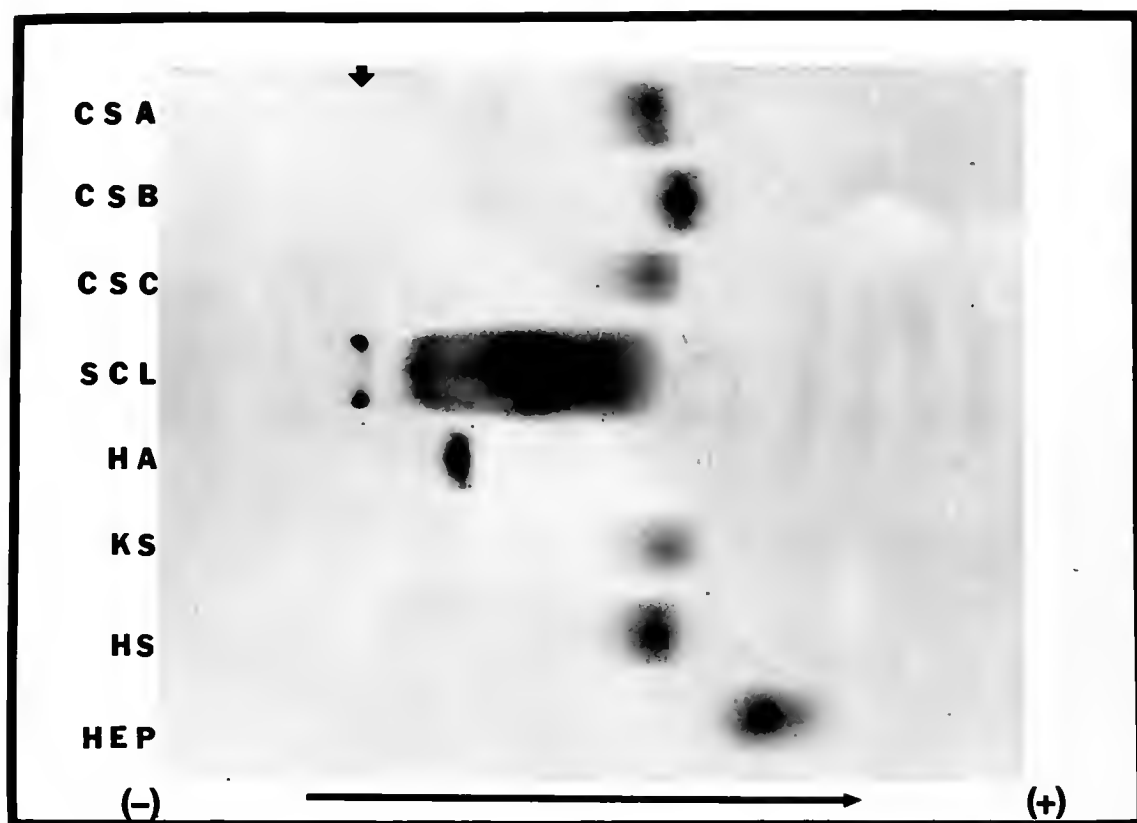


FIGURE 24. CELLULOSE ACETATE ELECTROPHORESIS OF MODERATE GLAUCOMATOUS SCLERA. Aliquot of STD = $0.5 \mu\text{g}$ and SCL = $0.5 \mu\text{g}$ in 0.1 M LiCl, 4.5 mA for 15 min . Abbr: CSA, CSB, CSC = Chondroitin A, B, C, SCL = sclera, HA = hyaluronic acid, KS = keratan sulfate, HS = heparan sulfate, Hep = heparin, Sm arrow = origin.

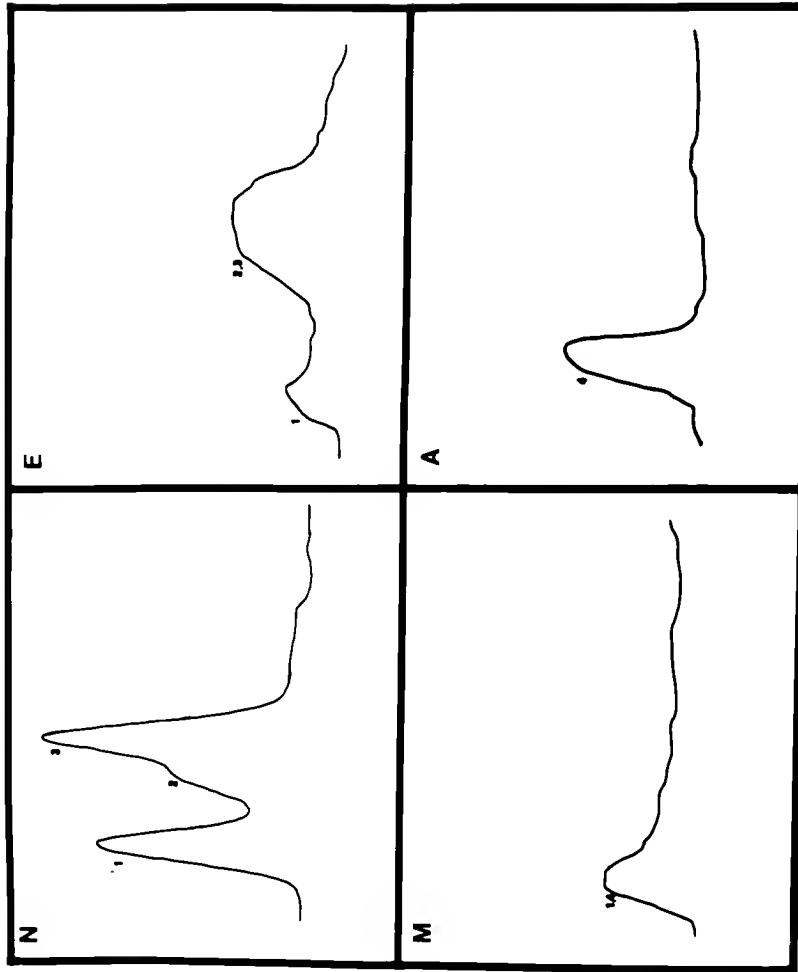


FIGURE 25. DENSITOMETRY RECORDINGS OF CELLULOSE ACETATE MEMBRANES OF NORMAL, EARLY, MODERATE, AND ADVANCED GLAUCOMATOUS TRABECULAR MESHWORK. 1 = HA, 2 = heparan sulfate, 3 = CSA - CSC, 4 = glycopeptide, N = normal, Relative % (1 = 34%, 2, 3 = 66%), E = early glaucoma, Relative % (1 = 25%, 2, 3 = 75%), M = moderate glaucoma, A = advanced glaucoma.

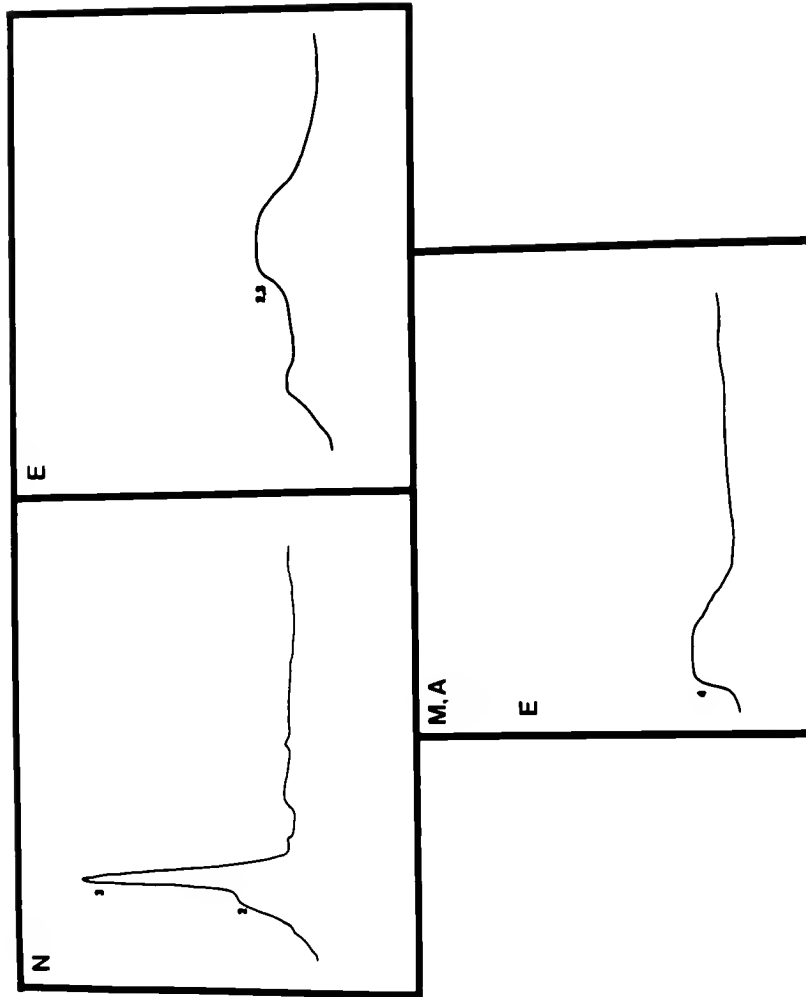


FIGURE 26. DENSITOMETRY RECORDINGS OF CELLULOSE ACETATE MEMBRANES AFTER ISOLATED TM GAGS WERE EXPOSED TO HYALURONATE LYASE. 2 = heparan sulfate, 3 = CSA - CSC, 4 = glycopeptide, N= normal, E = early glaucoma, M = moderate glaucoma, A = advanced glaucoma.

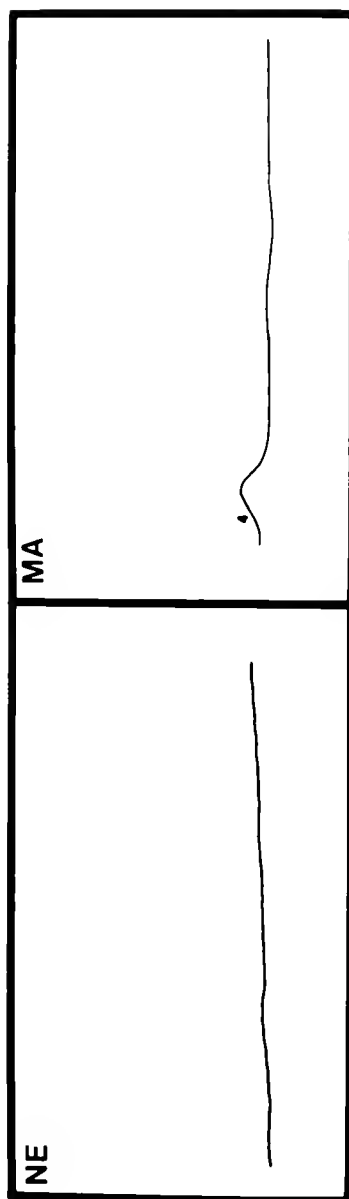


FIGURE 27. DENSITOMETRY RECORDINGS OF CELLULOSE ACETATE MEMBRANES AFTER ISOLATED TRABECULAR MESHWORK GAGS WERE EXPOSED CHONDROITIN TO ABC LYASE, HEPARITINASE, HEPARINASE AND KERATANASE. NE = normal and early glaucoma, MA = moderate and advanced glaucoma.

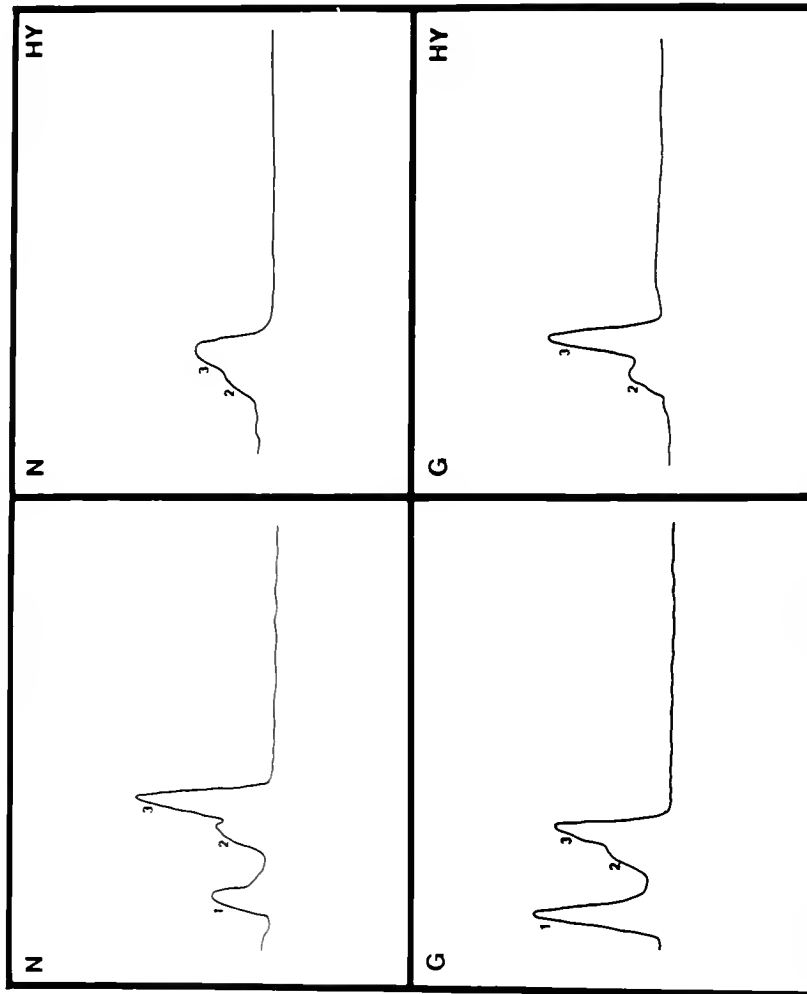


FIGURE 28. DENSITOMETRY RECORDINGS OF CELLULOSE ACETATE MEMBRANES OF NORMAL AND GLAUCOMATOUS IRIS-CILIARY BODY BEFORE AND AFTER HYALURONATE LYASE. 1 = HA, 2 = heparan sulfate, 3 = CSA - CSC, N = normal, Relative % (1 = 20%, 2 = 19%, 3 = 61%), G = glaucoma Relative % (1 = 46%, 2, 3 = 54%), HY = hyaluronidase.

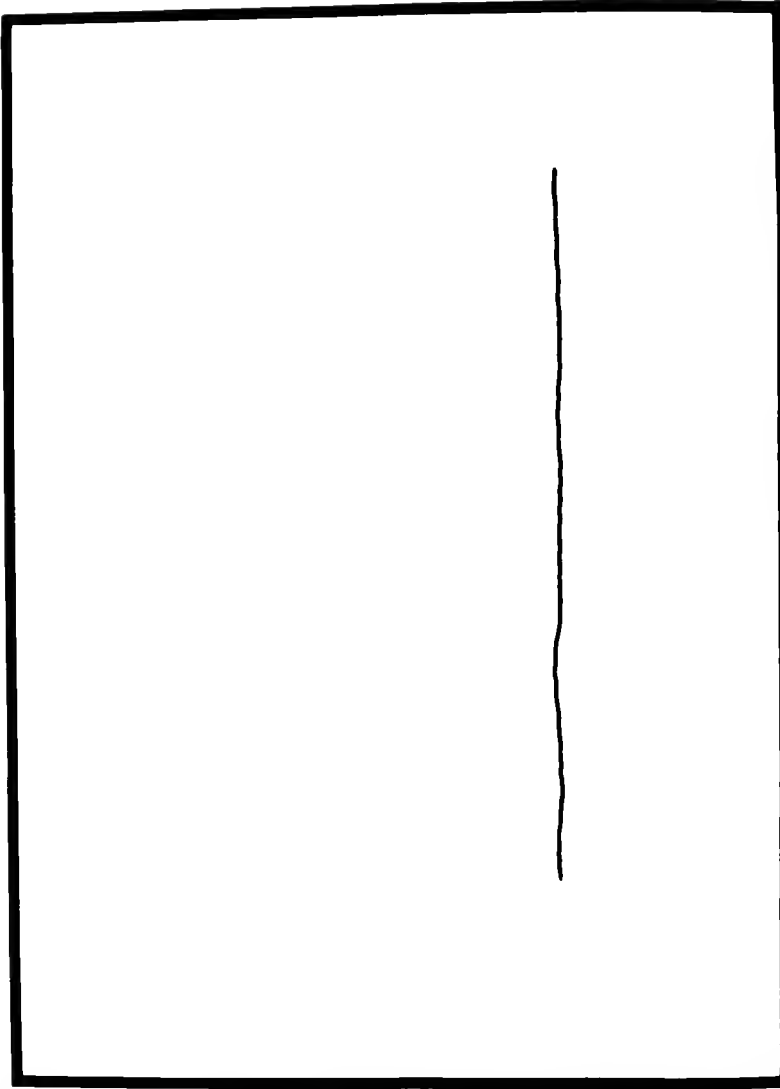


FIGURE 29. DENSITOMETRY RECORDING OF CELLULOSE ACETATE MEMBRANES OF NORMAL AND GLAUCOMATOUS IRIS-CILIARY BODY AFTER CHONDROITIN ABC LYASE, HEPARITINASE, HEPARINASE AND KERATANASE.

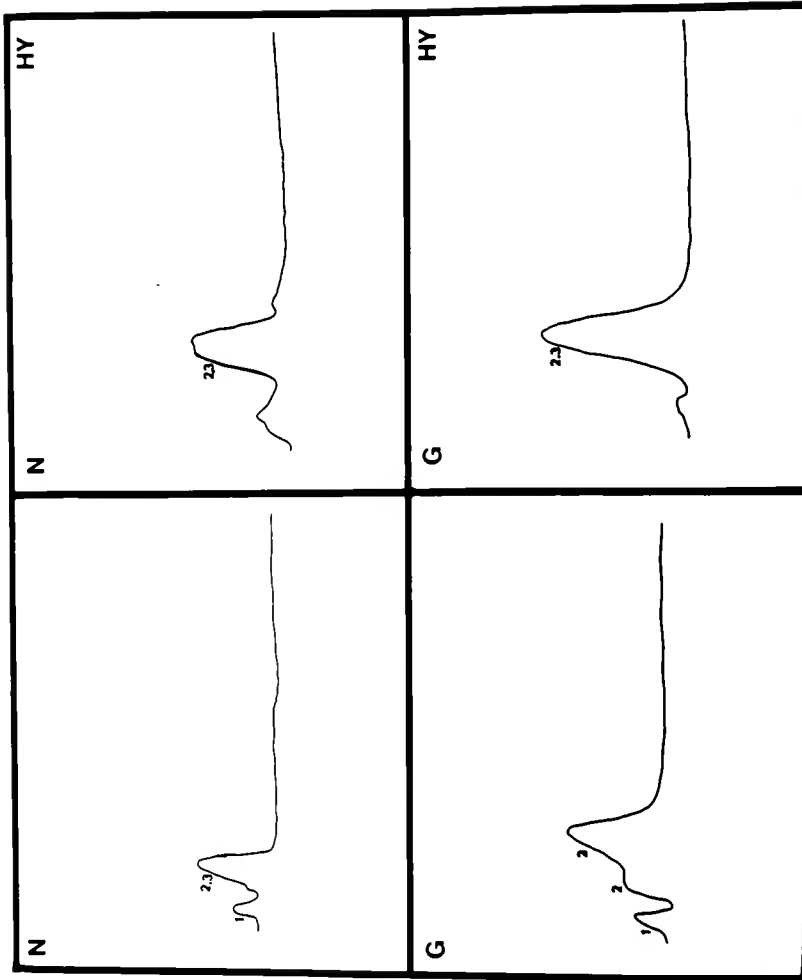


FIGURE 30. DENSITOMETRY RECORDINGS OF CELLULOSE ACETATE MEMBRANES OF NORMAL AND GLAUCOMATOUS SCLERA BEFORE AND AFTER HYALURONATE LYASE. 1 = HA, 2 = heparan sulfate, 3 = CSA - CSC, N = normal, Relative % (1 = 38%, 2, 3 = 62%), G = glaucoma, Relative % (1 = 29%, 2 = 27%, 3 = 44%), HY = hyaluronidase.

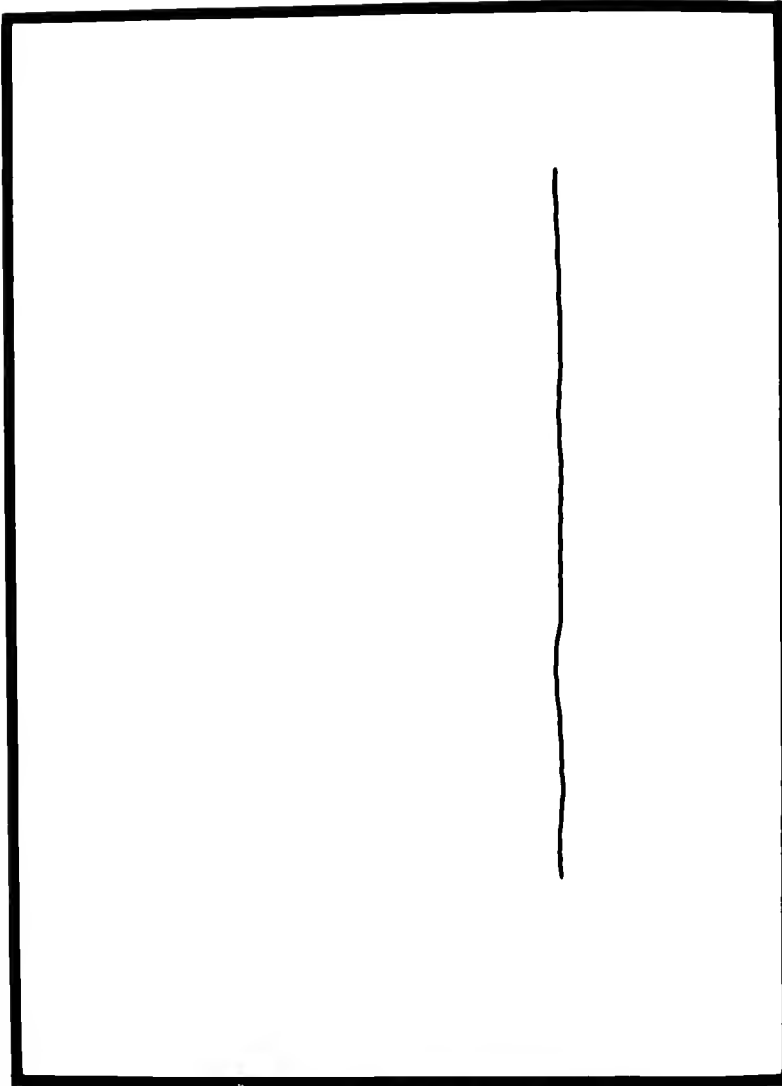


FIGURE 31. DENSITOMETRY RECORDING OF CELLULOSE ACETATE MEMBRANES OF NORMAL AND GLAUCOMATOUS SCLERA AFTER CHONDROITIN ABC LYASE, HEPARITINASE, HEPARINASE AND KERATANASE.

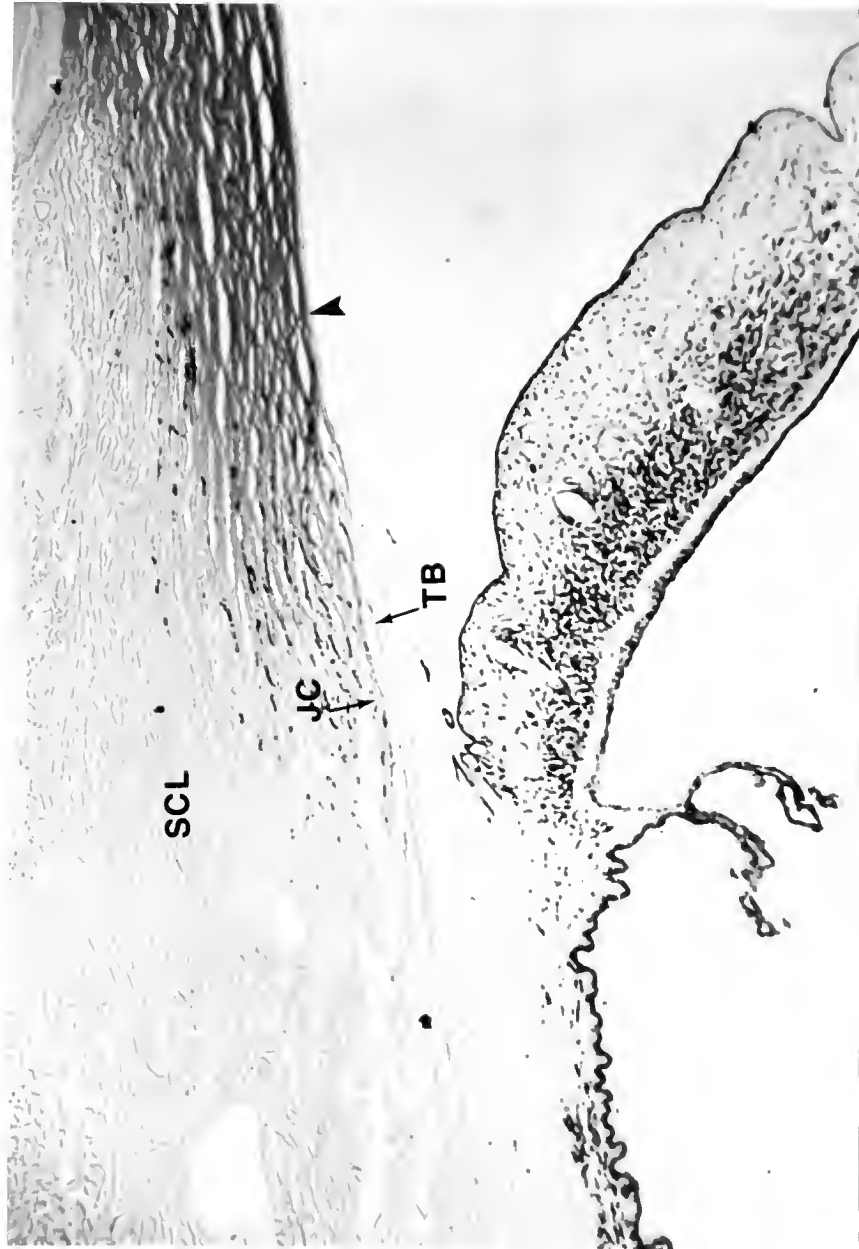


FIGURE 32. CANINE IRIDOCORNEAL ANGLE STAINED WITH ALCIAN BLUE. ARROW = Descemet's membrane, JC = juxtacanalicular, SCL = sclera, TB = trabecular beam.

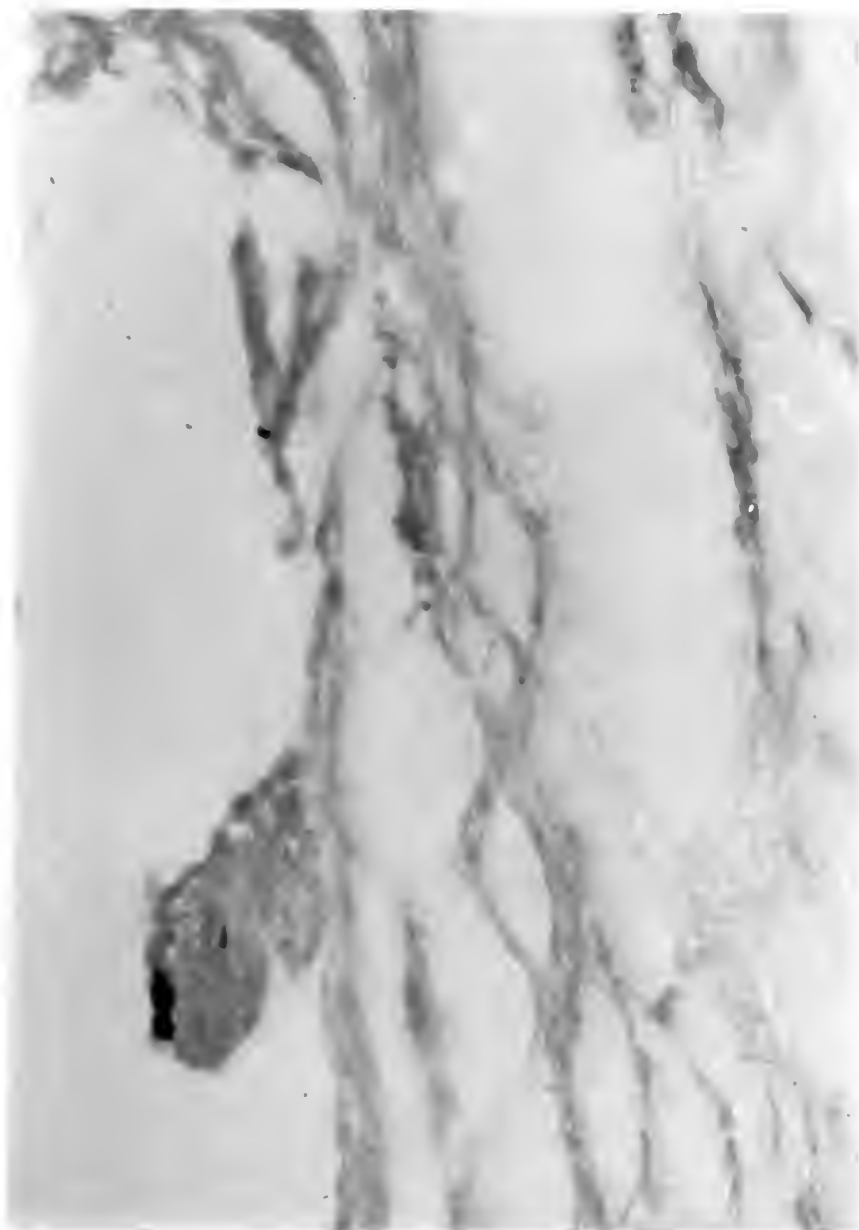


FIGURE 33. TRABECULAR MESHWORK BEAMS STAINED WITH ALCIAN BLUE.

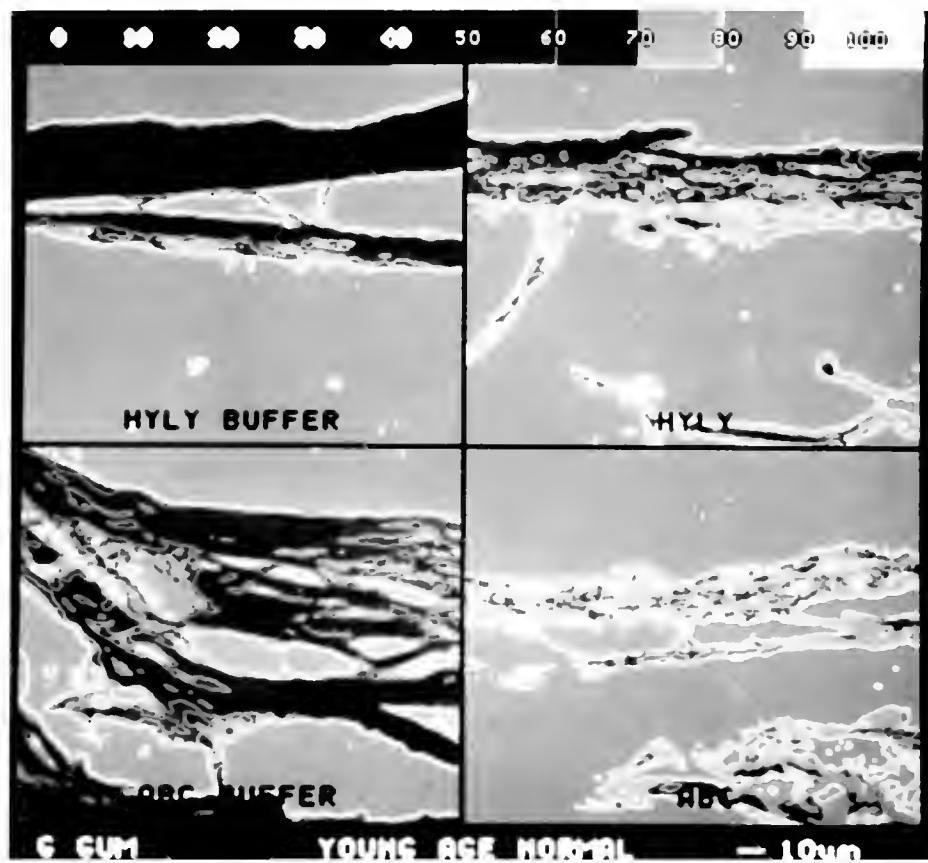


FIGURE 34. MICROGRAPH OF THE EARLY AGE NORMAL IRIDOCORNEAL ANGLE GENERATED BY THE ZEISS IMAGE PROCESSING SYSTEM. Scale at top represents percent transmission. Hyly = hyaluronate lyase, ABC = Chondroitin ABC lyase.

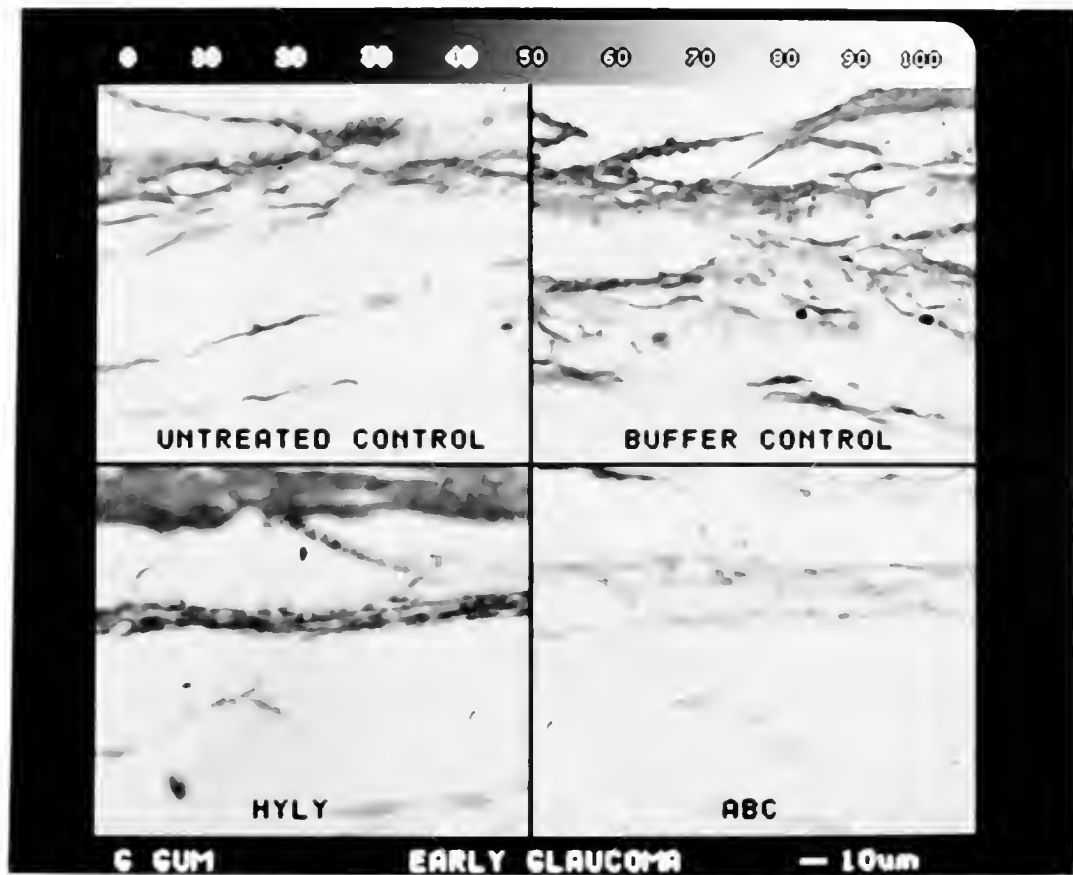


FIGURE 35. MICROGRAPH OF THE EARLY GLAUCOMATOUS IRIDOCORNEAL ANGLE GENERATED BY THE ZEISS IMAGE PROCESSING SYSTEM. Scale at top represents percent transmission. Hyly = hyaluronate lyase, ABC = chondroitin ABC lyase.

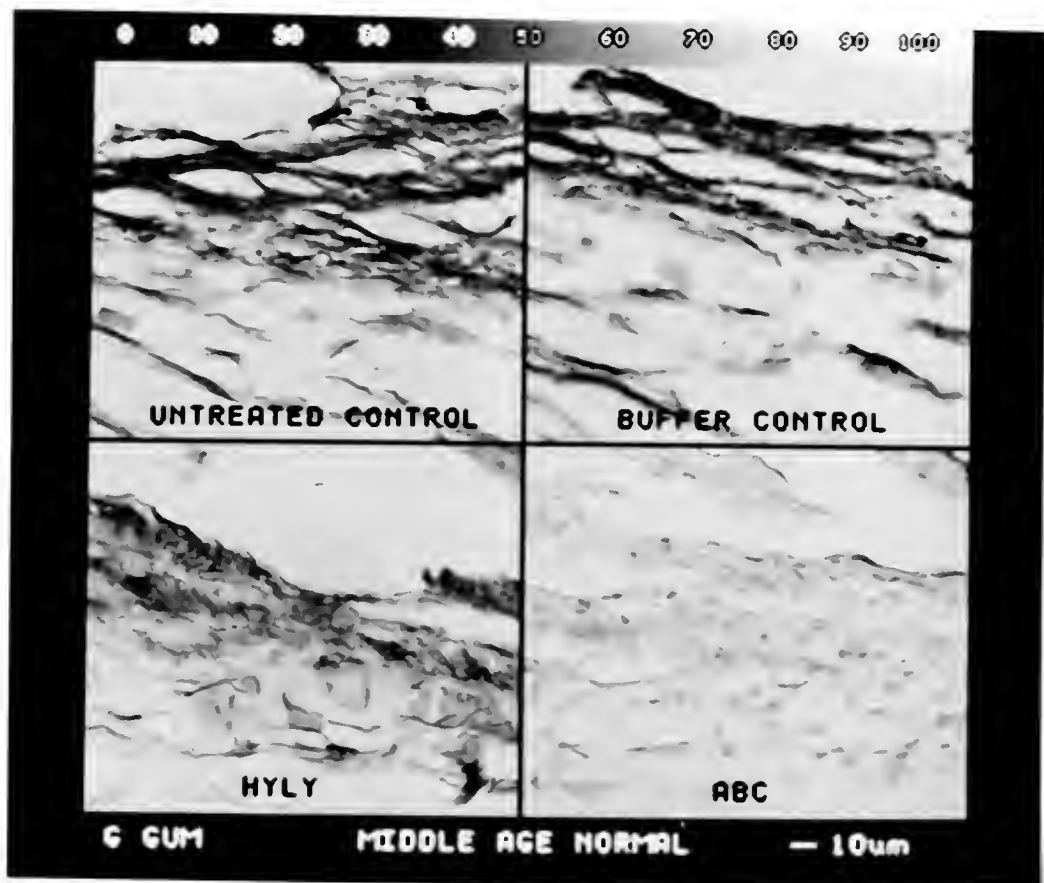


FIGURE 36. MICROGRAPH OF THE MODERATE AGE NORMAL IRIDOCORNEAL ANGLE GENERATED BY THE ZEISS IMAGING PROCESSING SYSTEM. Scale at top represents percent transmission. Hyly = hyaluronate lyase, ABC = chondroitin ABC lyase.

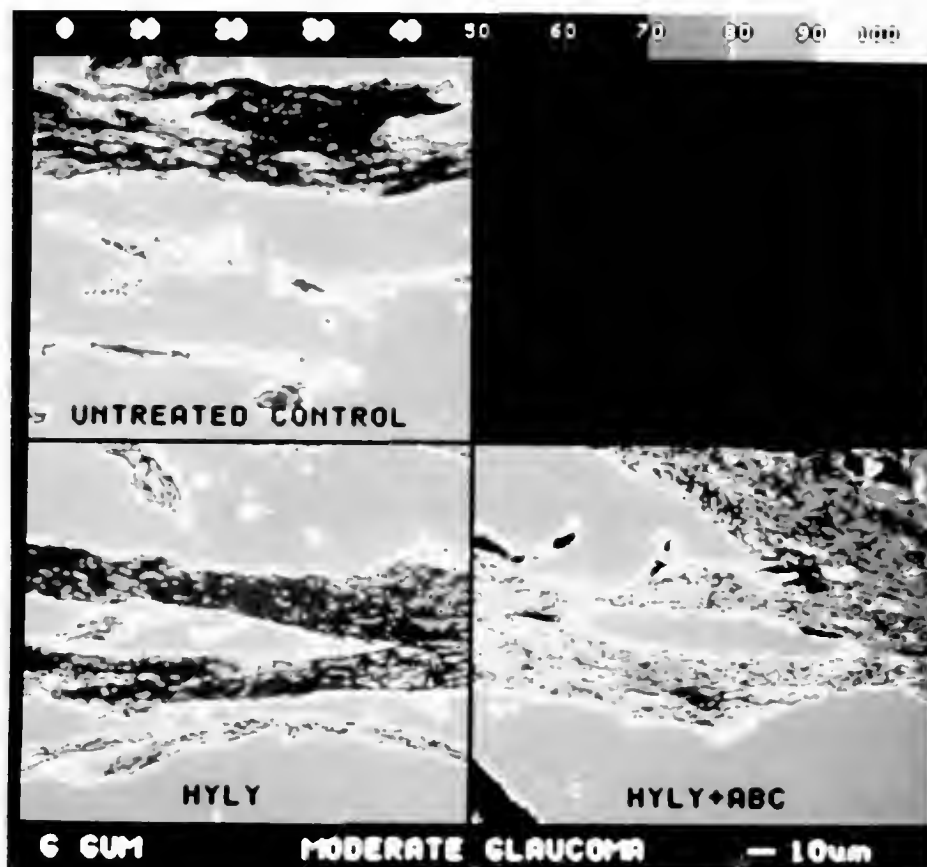


FIGURE 37. MICROGRAPH OF THE MODERATE GLAUCOMATOUS IRIDOCORNEAL ANGLE GENERATED BY THE ZEISS IMAGE PROCESSING SYSTEM. Scale at top represents percent transmission. Hyly = hyaluronate lyase, ABC = chondroitin ABC lyase.

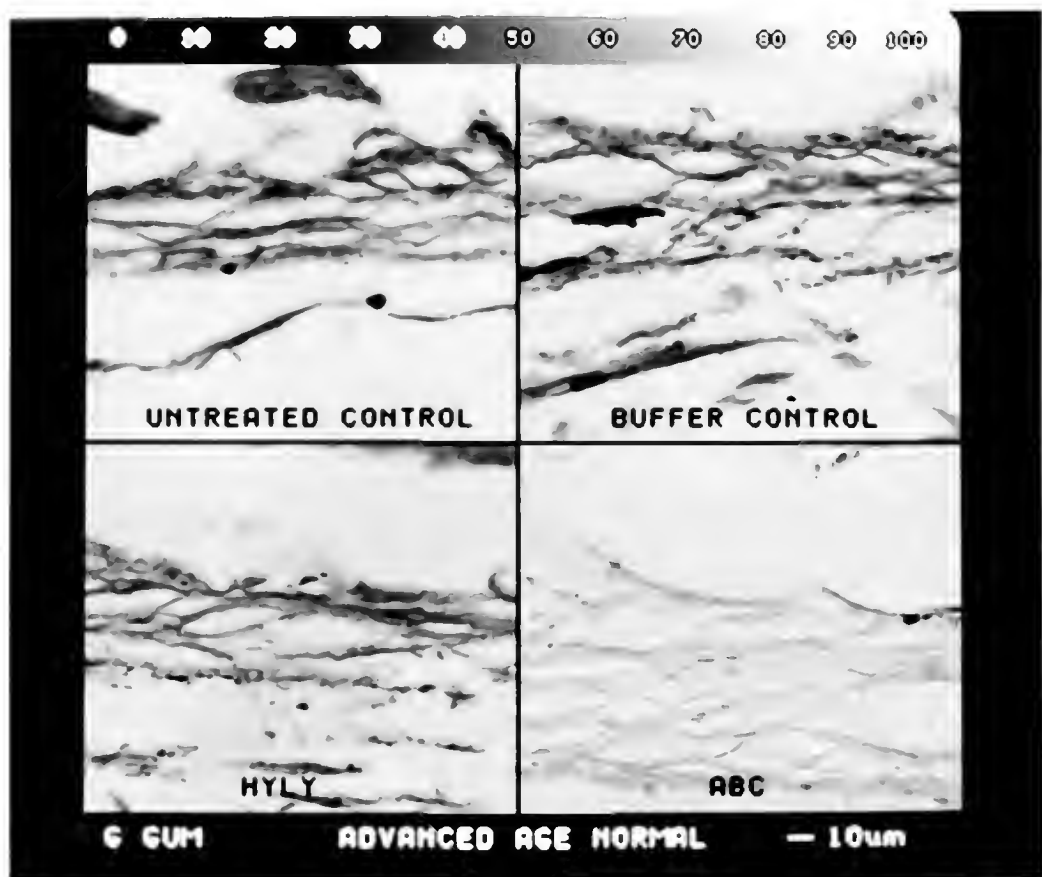


FIGURE 38. MICROGRAPH OF THE ADVANCED AGE NORMAL IRIDOCORNEAL ANGLE GENERATED BY THE ZEISS PROCESSING SYSTEM. Scale at top represents percent transmission. Hyly = hyaluronate lyase, ABC = chondroitin ABC lyase.

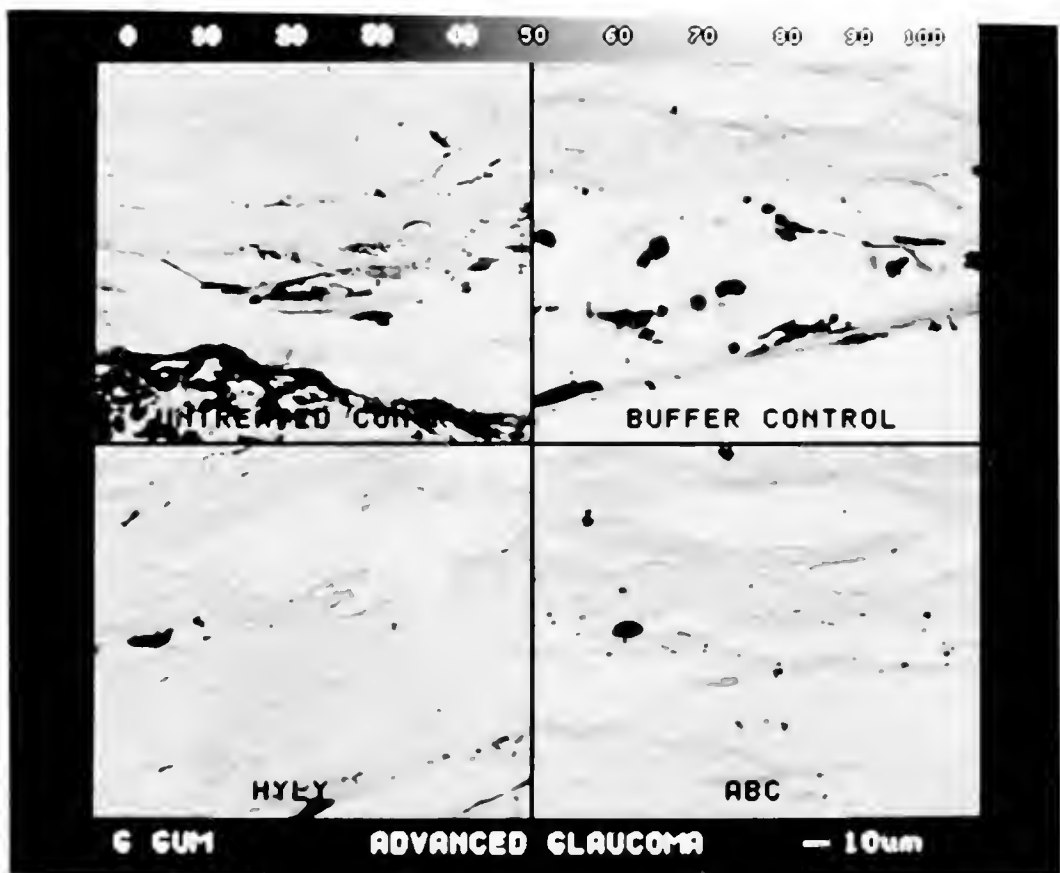


FIGURE 39. MICROGRAPH OF THE ADVANCED GLAUCOMATOUS IRIDOCORNEAL ANGLE GENERATED BY THE ZEISS IMAGE PROCESSING SYSTEM. Scale at top represents percent transmission. Hyly = hyaluronate lyase, ABC = chondroitin ABC lyase.

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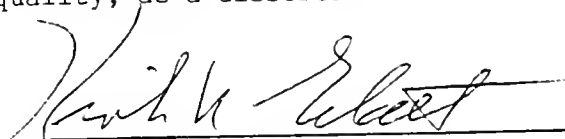
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BIOGRAPHICAL SKETCH

I was born in East Stroudsburg, Pennsylvania and attended Pen Argyl Area High School in Pen Argyl, Pennsylvania. In 1972 I graduated with a Bachelors of Science degree from Delaware Valley College of Science and Agriculture, Doylestown, Pennsylvania. My major was animal science--physiology and laboratory animal science. After graduation I accepted a position with the Dow Chemical Company in Midland, Michigan. After one year I accepted a position as a laboratory research assistant in the College of Veterinary Medicine at the University of Minnesota, St. Paul, Minnesota. In 1977, I received a Master of Science degree from the University of Minnesota, Minneapolis, Minnesota. My area of specialization was animal physiology, concentrating on electrophysiology of the retina and statistics. From 1977 to 1984 I was employed as an Assistant in Comparative Ophthalmology in the College of Veterinary Medicine, University of Florida, Gainesville, Florida. In 1984 I returned to graduate school, on a full time basis, as a Graduate Research Associate in the College of Veterinary Medicine. I received my Ph.D. degree (specializing in physiology, biochemistry and computer science) in Animal Science-Veterinary Science from the University of Florida, December 1986. I have authored or co-authored 48 scientific publications and a chapter in Veterinary Clinics of North America. I have also served as a consultant to other universities as well as private practitioners in establishing electrophysiological systems.

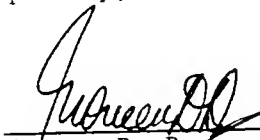
I have presently accepted a position as an Assistant Professor in the Department of Comparative Ophthalmology, College of Veterinary Medicine at the University of Florida where I plan to continue my research career in the biochemical aspects of glaucoma.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.



Kirk N. Gelatt, Chairman
Professor of Animal Science and
Veterinary Medicine

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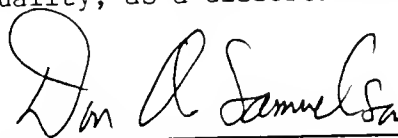
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Assistant Research Scientist
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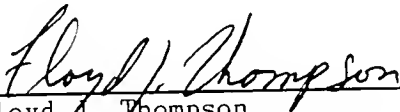
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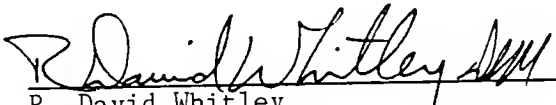
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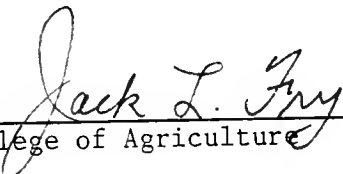
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This dissertation was submitted to the Graduate Faculty of the College of Agriculture and to the Graduate School and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

December 1986



Dean, College of Agriculture

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